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The role of 1,25 Dihydroxyvitamin D3 and other calcitrophic hormones
as immunoregulatory agents.

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Thesis submitted for the degree of Doctor of Philosophy (Ph.D.)

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Dedicated to Lorna and Samantha

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Abbreviations

B - cells	B - lymphocytes
BSA	Bovine serum albumin
b(1 - 35)PTH	Bovine (1 - 35) parathyroid hormone
CT	Calcitonin
CD4 +ve	helper T - cells with clonally derived 4 antigen
CD8 +ve	suppressor T - cells with clonally derived 8 antigen
Ci	Curie
Con A	Concanavalin A
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetra - acetate
ELISA	Enzyme - linked immunosorbant assay
FCS	Foetal calf serum
HCL	Hydrochloric acid
HEPES	N - [2 - Hydroxyethyl] piperazine - N - [2ethanesulphonic] acid
HGF	Hybridoma growth factor
HRP	Horse radish peroxidase
H ₂ SO ₄	Sulphuric acid
IFN-γ	Interferon - γ
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1	Interleukin - 1
IL-2	Interleukin - 2
IL-2R	Interleukin - 2 receptor
IL-3	Interleukin - 3
IL-4	Interleukin - 4
IL-5	Interleukin - 5

IL-6	Interleukin - 6
IL-7	Interleukin - 7
IL-8	Interleukin - 8
IL-9	Interleukin - 9
IL-10	Interleukin - 10
IMDM	Iscoves modified Dulbeccos Medium
i.units	International units
kD	kilodalton
MEM	Minimum essential medium
mRNA	Messenger ribonucleic acid
MTT	3 - (4,5 - Dimethylthiazol - 2 - yl) - 2,5 - diphenyl tetrazolium bromide
Mφ	Macrophage
OD	Optical density
OPD	o - Phenylenediamine
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PDGF	Platlet derived growth factor
PGE ₂	Prostaglandin E ₂
PHA	Phytohaemagglutinin
PHS	Pooled human serum
PPO	2,5 Diphenyloxazole
PTH	Parathyroid hormone
PWM	Pokeweed mitogen
RNA	Ribonucleic acid
RPMI 1640	Rosewell Park Memorial Institute 1640 medium
sCT	Salmon calcitonin
sIL-2R	Soluble interleukin - 2 receptor
T - cells	T - lymphocytes

TCA	Trichloroacetic acid
TCGF	T - cell growth factor
T _H	T - helper cell
TNF α	Tumour necrosis factor α
T _s	T - suppressor cell
VDR	Vitamin D receptor
1 α OHase	1 α hydroxylase
1,25(OH) ₂ D ₃	1,25 dihydroxyvitamin D ₃
24,25(OH) ₂ D ₃	24,25 dihydroxyvitamin D ₃
25(OH)D ₃	25 hydroxyvitamin D ₃
³ H-thy	Tritiated thymidine

SUMMARY

The thesis should be read in conjunction with the addendum that follows the summary.

Over the years it has been well documented that there is a connection between the immune system and the endocrine system certainly as far as vitamin D was concerned. The major breakthrough came when vitamin D receptors were demonstrated on various cells whose principal task was to mount an immune response. 1,25 Dihydroxyvitamin D₃ (1,25(OH)₂D₃), the active metabolite of vitamin D, was demonstrated to inhibit cell proliferation and to inhibit interleukin - 2 (IL-2) activity (Tsoukas, 1984). In this thesis the roles of 1,25(OH)₂D₃ and other calciotropic hormones as immunoregulatory agents are addressed.

The results presented in this thesis demonstrate that the effects of 1,25(OH)₂D₃ on the immune system are both genuine and unique. The other calciotropic hormones studied (24,25 dihydroxyvitamin D₃(24,25(OH)₂D₃), 25 hydroxyvitamin D₃ (25(OH)D₃), parathyroid hormone (PTH) and calcitonin (CT)) did not have a significant effect on either cell proliferation or IL-2 activity. Although it had been previously demonstrated that sex hormones (oestrogen, progesterone and dihydrotestosterone) could inhibit the immune response this effect was demonstrated to be different from the inhibition by 1,25(OH)₂D₃ in the immune system. 1,25(OH)₂D₃ was demonstrated to have little effect on the other cytokines that were prominent in the system under review. 1,25(OH)₂D₃ had no significant effect on either interleukin - 1 (IL-1) or interleukin - 6 (IL-6).

The capacity of 1,25(OH)₂D₃ to act as a differentiation factor has been well documented. It was conceivable that 1,25(OH)₂D₃ acts as a differentiation factor in the immune system. The possible target would be the suppressor cells (Ts) and the 1,25(OH)₂D₃ could promote the differentiation of these cells. This scenario was not demonstrated but the effect of 1,25(OH)₂D₃ was shown to be mediated through the helper T-cells (T_H).

1,25(OH)₂D₃ was demonstrated to increase the concentration of the soluble interleukin-2 receptor (sIL-2R) at concentrations in excess of 10⁻¹¹ M. This increase was a characteristic 'all or nothing' response. It is significant that the switch from low sIL-2R to high sIL-2R occurs between 10⁻¹¹ M and 10⁻¹⁰ M which is the physiological range of 1,25(OH)₂D₃. It was also demonstrated that 1,25(OH)₂D₃ inhibited the message for both interleukin - 2 (IL-2) and the interleukin - 2 receptor (IL-2R) at 10⁻¹⁰ M but that inhibition of the message for both was not seen at 10⁻⁷ M.

Taken together the work in thesis suggests that 1,25(OH)₂D₃ inhibits IL-2 activity through two distinct mechanisms. 1,25(OH)₂D₃ acts as a 'classic steroid hormone' between 0 M and 10⁻¹¹ M in that it inhibits IL-2 and indeed IL-2R at the genomic level by directly inhibiting the amount of message for these proteins. Between the concentrations 10⁻¹⁰ M and 10⁻⁷ M the 1,25(OH)₂D₃ increases the concentration of sIL-2R. The increase in sIL-2R acts as an antagonist to IL-2 and effectively 'mops' up the IL-2 subsequently produced thereby sustaining the inhibition of the IL-2 activity.

ADDENDUM

The thesis confirms that $1,25(\text{OH})_2\text{D}_3$ inhibits both IL-2 activity and lymphocyte proliferation. However, under the conditions of the system no inhibitory effect was demonstrated by the other vitamin D metabolites ($25(\text{OH})\text{D}_3$ or $24,25(\text{OH})_2\text{D}_3$) or by the calciotropic hormones PTH and CT. The effect of calcitonin and parathyroid hormone on lymphocyte proliferation has to be interpreted carefully as the system was set up to look for inhibition. It is important to realise that nothing can be concluded about possible enhancement of response. An inhibitory response was obtained by sex hormones in the system but this effect produced a characteristic U - shaped curve which was distinct from the effect observed with $1,25(\text{OH})_2\text{D}_3$.

Throughout this thesis the measurement of IL-2 activity was carried out using the IL-2 dependent cell line, HT-2. This cell line was derived from a single cell that has the characteristics of a murine helper T - cell (Watson, 1979). The HT-2 cells were demonstrated to be responsive to murine, rat and human IL-2 and had the advantage of not being responsive to the T - cell mitogens, PHA and Con A (Watson, 1979). The HT-2 cells were demonstrated to be dependent on IL-2, withdrawal of IL-2 resulting in cell death within 2 days (Doyle, 1985) and this was confirmed (data not shown). As a consequence of measuring cytokines in cell supernatants it is possible that other cytokines presently defined or as yet undefined may stimulate HT-2 cells. This was felt to be less likely as two other bioassays for measuring IL-2 produced similar results (see section 4.3.). Further confirmation could have been obtained by observing the effect of a neutralising antibody against IL-2 on the IL-2 containing supernatants this antibody should block the IL-2 response whereas antibodies against other cytokines should have no effect.

It was possible that the assays may have been affected by IL-2, vitamin D metabolites or sex hormones in the FCS. The batch of FCS was constant throughout the studies therefore any effect of these factors would be consistent. The concentration of FCS was only 2 % thereby keeping any effect to a minimum. The biological half - life of the vitamin D metabolites make it unlikely that they could contribute significantly to the results presented. It has been reported that $1,25(\text{OH})_2\text{D}_3$ has a biological half - life of

1.5 days (Kanis, 1977). IL-2 is normally undetectable in serum and also has a short half-life. The sex hormones in FCS were not expected to be high as these hormones are associated with maturity. The vitamin D metabolites and sex hormones present in the serum used in the IL-2 production experiments however, could have been removed altogether by charcoal stripping the serum. The range of $1,25(\text{OH})_2\text{D}_3$ concentrations studied (0 to 10^{-7}M) was shown to have no effect on the culture of the HT-2 cells, LBRM TG6 and B9 (data not presented).

The majority of the work was carried out on peripheral blood mononuclear cells (PBMCs) obtained from normal males in order to minimise variation. The only exception to this was the work on the sex hormones. In all the work involving sex hormones the population was mixed. PBMCs from both males and females were cultured in the presence of all three sex hormones (oestrogen, progesterone and dihydrotestosterone) and no difference in response with respect to sex was observed. The effect of sex hormones on lymphocyte proliferation was carried out on five females and four males whereas the effect of sex hormones on IL-2 activity was carried out on two females and two males.

The IL-2 activity obtained from PBMC's varied from individual to individual. However, this variability was not sufficiently large that it could be accounted for by gene duplication in certain individuals as reported for some other cytokines (e.g. $\text{TNF } \alpha$, (Jongeneel, 1991)). IL-2 has been demonstrated to be a product of a single gene consisting of four exons and three introns on the long arm of chromosome 4 in humans (Holbrook, 1984).

The paired Wilcoxon test (Wilcoxon, 1945) was the statistical method used throughout the thesis because the small sample size did not allow the assumption that the distribution was normal. Nonparametric statistics were thus favoured. This statistical technique requires a minimum of seven samples (Lehmann, 1975) hence this was the number used. As a consequence in situations where there were less than seven samples in a study, statistics were considered inappropriate. All analyses were classified as significant when the p value was less than 0.05 as is usual statistical practice but were

not graded below this level e.g. $p < 0.001$. This could have underestimated the significance of the results especially those showing a gradual but increasing effect as demonstrated by the effect of $1,25(\text{OH})_2\text{D}_3$ on IL-2 activity (p 126). The grading of significance could have shown that the effect of $1,25(\text{OH})_2\text{D}_3$ on IL-1 and IL-6 activities were less significant than the effect of $1,25(\text{OH})_2\text{D}_3$ on IL-2 and hence the one point that did achieve significance at the level of $p < 0.05$ would be discredited (see p 149 and p 161). An alternative to the Wilcoxon test would have been the analysis of variance test. This assesses whether the variation among groups is significantly greater than that which would be expected given the variation within a group. When this analysis was carried out in retrospect no significant differences were obtained in the IL-1 and IL-6 data whereas the differences remained significant in the IL-2 data.

Chapter 8 in this thesis looks at the effects of $1,25(\text{OH})_2\text{D}_3$ on the mRNA for IL-2 and IL-2R. The data suggests a reduction in the mRNA for IL-2 and IL-2R after incubation with 10^{-10} M $1,25(\text{OH})_2\text{D}_3$ at 20 hours. However, although the pattern obtained in figure 8.2.1. looks quite convincing there are two areas of concern which require reconsideration of some of the conclusions declared on pages xxv, 199 and 203. The amount of RNA loaded onto the gel should have been the same as was suggested by the concentration of the RNA calculated by nucleic acid dip sticks and by the appearance of the RNA on the preparatory gel, but in fact there was no adequate control. The solution to this problem would be to strip the blots and reprobe with an *abl* or β - actin probe. This would verify that there was an equal amount of mRNA in each lane on the gel. A second problem occurs when the position of the bands are considered: the size of the mRNA coding for the IL-2 (about 465 bases) should be considerably smaller than the mRNA coding for the IL-2R (about 816 bases). This size difference was not observed in the blots obtained. The position of the band on the gel makes it unlikely that it was the IL-2 probe that was responsible for this discrepancy. Rigby (Rigby, 1987) has also demonstrated that $1,25(\text{OH})_2\text{D}_3$ inhibits the mRNA for IL-2 at 20 hours. This raises questions about the specificity of the probe for the IL-2R. The solution would be to

obtain another probe for the IL-2R and reprobe the blot. It would also be advisable to include an RNA ladder to size the bands on the blot.

The postulate that a two mechanism system exists can be clarified in several ways. Preliminary experiments performed after the thesis was completed suggested that the $1,25(\text{OH})_2\text{D}_3$ inhibition was only partially reversed by addition of IL-2. At the higher concentrations of $1,25(\text{OH})_2\text{D}_3$ (10^{-8}M and 10^{-7}M) there is still a slight inhibition of lymphocyte proliferation which could possibly be accounted for by a second mechanism. In order to ascertain whether or not the $1,25(\text{OH})_2\text{D}_3$ is regulating the IL-2 gene the next stage would be to search for and possibly locate a vitamin D acceptor site. This work would involve searching the DNA sequence upstream from the IL-2 gene for the presence of a consensus sequence similar to the one known to act as an acceptor site for the vitamin D in other systems.

Nevertheless, the thesis clearly demonstrates that $1,25(\text{OH})_2\text{D}_3$ inhibits both IL-2 activity and lymphocyte proliferation and that the other calciotropic hormones tested were unable to do so. The inhibition by $1,25(\text{OH})_2\text{D}_3$ was demonstrated to be different from the effect documented for the sex hormones. $1,25(\text{OH})_2\text{D}_3$ was shown to increase the concentration of sIL-2R present in cell supernatants thus suggesting a double mechanism of action of $1,25(\text{OH})_2\text{D}_3$ on cell proliferation i.e. by reducing IL-2 production and by increasing soluble IL-2 receptor production.

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CHAPTER 1.

GENERAL INTRODUCTION.

1.1 INTRODUCTION.

Vitamin D in its active form is an important regulator of extracellular calcium homeostasis and has therefore a fundamental role in the physiology of bone. Historically, this importance in mineral metabolism was highlighted by the clinical condition of rickets - osteomalacia where there was usually a deficiency of, or a resistance to the action of vitamin D. However, in more recent years it has become apparent that the physiological role of vitamin D may extend to promotion of cell differentiation and immunoregulation.

The importance of these further actions of vitamin D has led to further pharmacological applications. Holick's group and others have successfully used such active metabolites of vitamin D in the treatment of psoriasis (Smith, 1988), and these metabolites have also been used as an adjunct to chemotherapy in the treatment of lymphoma (Cunningham, 1985). The treatment of leukaemias with vitamin D metabolites has limitations in that the effective therapeutic concentrations are 10 to 100 times higher than their physiological serum levels (Porteous, 1987). Gepner has also suggested that vitamin D may be potentially useful in the management of rheumatoid arthritis (RA) (Gepner, 1989).

A full appreciation of these additional roles of vitamin D and its metabolites on haematopoietic cell differentiation and on the immune system requires firstly an understanding of physiological roles of vitamin D, secondly its biochemistry and metabolism and thirdly an appreciation of the role of cytokines in the immune system.

1.2 VITAMIN D PHYSIOLOGY.

1.2.1 Is vitamin D a vitamin ?

The classical definition of a vitamin was that it is a trace dietary constituent required to effect normal physiological function. Vitamin D, however, is really a misnomer as higher animals can synthesise calciferol in skin when exposed to ultraviolet light (u.v.) - usually in the form of sunlight. This in effect means that vitamin D is only a true vitamin when the animal is deprived of sunlight.

As in the case of other true vitamins, absence of vitamin D is associated with specific biological abnormalities. These abnormalities may affect the organism as a whole (retardation of growth), or particular organs (development of rickets - osteomalacia by the skeletal system), or particular cell types (the columnar epithelial and/or goblet cells of the intestinal mucosa), and ultimately be manifest at the subcellular level (alteration of the morphology of kidney mitochondria) (Norman, 1979).

There are several theories as to the first appearance of the vitamin D deficient state in man. On the basis of palaeontological evidence both Solecki (Solecki, 1971 a,b) and Loomis (Loomis, 1967) postulated that Neanderthal man's stooped appearance may in fact be due to vitamin D deficiency as opposed to a "low" evolutionary status. Kjerrulf (Kjerrulf, 1922) claimed that rickets could be observed in human skeletons from the Neolithic age (c.8000 B.C.).

Although there have been suggestions of afflictions similar to the clinical symptoms of rickets in the archaeological records of Egyptians (c.1500 B.C.), and in the writings of Soranus of Ephesus (Findlay, 1917 and Hess, 1929), the first precise written definition of rickets was credited to both Whistler (Whistler, 1645) and Glisson (Glisson, 1650 a,b). After independent publication of their description of rickets it became a commonly recognised disease entity .

A major breakthrough occurred between 1919 and 1921, when in a comprehensive series of experiments using rachitic pups fed accurately defined diets Mellanby demonstrated that a nutritional factor possessing antirachitic activity existed (Mellanby, 1921). Mellanby went on to suggest that this antirachitic factor was the fat soluble vitamin A. Furthermore, he demonstrated that cod-liver oil was an excellent antirachitic agent thus confirming a piece of rural wisdom that had been around for many years. However, McCollum et al (McCollum, 1922) demonstrated that the essential factor was not vitamin A and therefore proposed that the antirachitic substance be named vitamin D. The critical observation that supported a distinct antirachitic factor in cod-liver oil was that the proposed agent could survive both aeration and heating (100 °C for 14 hours) whereas under these conditions vitamin A was inactivated.

At this time it also became apparent that ultraviolet light had an important role to play in the aetiology and cure of rickets. This feature was demonstrated by Huldschinsky (Huldschinsky, 1919, 1919-1920), who showed that u.v. rays were effective in increasing the degree of calcification of the epiphysis of rachitic children. This observation was confirmed in an animal model.

The work of Goldblatt and Soames (Goldblatt, 1923) made a connection between a molecular species (in cod-liver oil) responsible for the antirachitic activity and u.v. light. They demonstrated that if livers were excised from rachitic rats and were subjected to irradiation with u.v. light, and subsequently if these irradiated livers were then fed to other rachitic rats, the vitamin D deficiency was corrected in these rats. Steenbock and Black (Steenbock, 1924) showed that irradiated food possessed antirachitic properties. Both groups were responsible for the conclusion that ultraviolet light played a critical role in producing vitamin D from a provitamin present in the skin.

Both Hess and Weinstock (Hess, 1925 a,b,c) and Steenbock and Black (Steenbock, 1925) confirmed that antirachitic activity could be generated by u.v. irradiation of various foods. They also fractionated the antirachitic factor. This factor was confined to the nonsaponifiable fraction and exclusively resided in the phytosterol or cholesterol fraction. Although both groups believed that they were studying the same factor, Steenbock and Black had produced vitamin D₂ from the irradiation of the ergosterol and Hess and Weinstock had generated vitamin D₃ from the irradiation of skin. Having made the connection between antirachitic activity, ultraviolet light and sterols - in particular 5,7-unsaturated sterols - it was now possible to characterise vitamin D.

1.2.2 The structure of vitamin D.

The identification of the chemical structure was the result of the simultaneous efforts of both Windaus et al (Windaus, 1932) and Askew et al (Askew, 1932). Vitamin D₃ was chemically characterised in 1936 by Windaus et al (Windaus, 1936). The antirachitic sterol was actually a secosteroid formally known as 9(10) seco cholesta 5,7,10(19)-dien-3B-ol. Seco-steroids are those in which one of the rings has undergone fission by breakage of a carbon-carbon bond (figure 1.2.1). The seco - nature of the vitamin was confirmed by X-ray crystallography (Crowfoot, 1948; Crowfoot - Hodgkin, 1963). Vitamin D₃ or cholecalciferol was the naturally occurring form of the vitamin in animals and was normally derived by exposure of 7 dehydrocholesterol, present in the skin, to sunlight. On the other hand, vitamin D₂ or ergocalciferol was produced through ultraviolet irradiation of the sterol ergosterol found in yeasts and fungi.

The metabolic pathway for Vitamin D

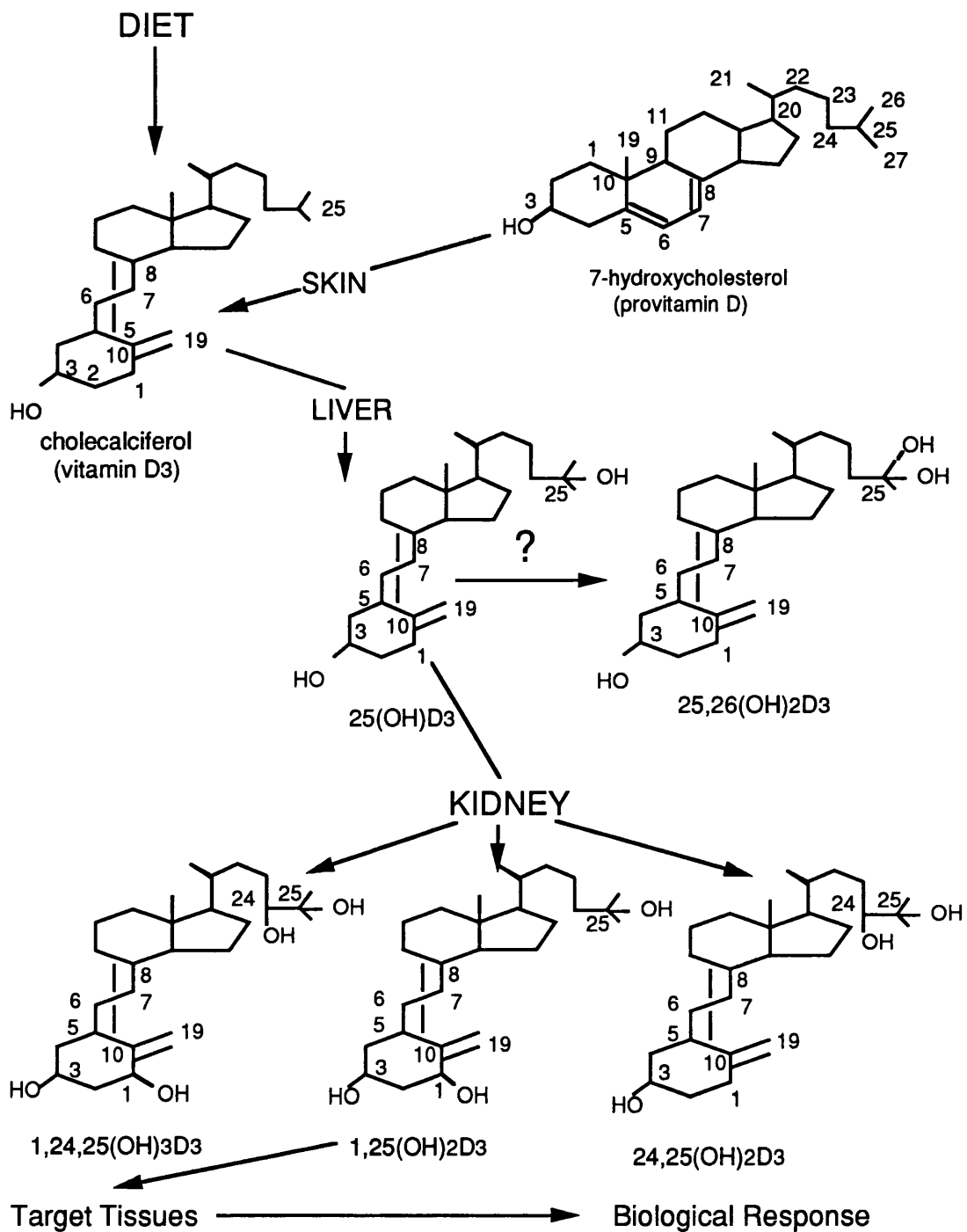


FIGURE 1.2.1.

The metabolic pathway for the production of 1,25 dihydroxyvitamin D₃ from either the photoconversion of 7 - dehydrocholesterol or from the diet.

In the early years of vitamin D research it became evident that there was a time lag between administration of vitamin D and a physiological response (Irving, 1944). This lag was probably as much to do with the use of crude end points as anything else. However, with the development of radioisotopes it became apparent that this lag was a genuine phenomenon. Raoul and Gounelle (Raoul, 1958) and Sallis and Holdsworth (Sallis, 1962) speculated that a metabolic conversion of vitamin D₃ to an active form was necessary. Of course it could have been that the lag was due to the slow absorption and transportation of the vitamin or perhaps it reflected an induction process involving expression of genetic information.

1.2.3 Vitamin D metabolites.

During the 1960's and 70's contributions from several laboratories unravelled the complex metabolic pathway for the production of the biologically active form of vitamin D. The development of randomly labelled tritiated vitamin D₂ and vitamin D₃ with high specificity by Norman and De Luca (Norman, 1963) allowed the detection of several metabolites that possessed antirachitic activity. These metabolites have now been characterised.

The important metabolites in blood were 25-hydroxyvitamin D₃ (25(OH)D₃) , 24,25 dihydroxyvitamin D₃ (24,25(OH)₂D₃) and 1,25 dihydroxyvitamin D₃ (1,25(OH)₂D₃). The human plasma concentrations of these metabolites were 25(OH)D₃ (10⁻⁸ M - 10⁻⁷ M , 15 - 30ng/ml), 24,25(OH)₂D₃ (10⁻¹⁰ M - 10⁻⁹ M, 1 - 3 ng/ml) and 1,25(OH)₂D₃ (10⁻¹¹ M - 10⁻¹⁰ M, 30 - 40 pg/ml). At present there are a further five daughter metabolites of vitamin D₃, none of which have been assigned a biological function. The most potent metabolite with regard to biological function in both the intestine and in the bone is 1,25 dihydroxyvitamin D₃.

Whether vitamin D₃ is derived from the diet or by photochemical conversion of 7-hydroxycholesterol, the next step is conversion to 25(OH)D₃. This metabolic conversion takes place principally in the liver and is catalysed by 25-hydroxylase located in hepatic mitochondria. The enzyme is regulated by a negative feedback mechanism. Vitamin D-25 Hydroxylase activity was also found in other tissues such as intestine and kidney (Tucker, 1973).

The circulating 25(OH)D₃ becomes a substrate for the renal enzymes, 25(OH)D₃-1-hydroxylase and 25(OH)D₃-24-hydroxylase. These enzymes were located in the mitochondrial fraction of the kidney cortex (Fraser, 1970) and each enzyme can be described as a classical mixed function steroid hormone hydroxylase (Ghazarian, 1973). The enzymes have at least three components, namely a flavoprotein (an iron - sulphur protein), renal ferredoxin, and a cytochrome P450. Together these components facilitate the transfer of electrons from NADPH to the cytochrome P450 and ultimately the incorporation of molecular oxygen into a hydroxyl moiety at position 1 or 24.

1.2.4. Regulation of 1,25(OH)₂D₃.

It is now well established that 1,25(OH)₂D₃ is the vitamin D metabolite that plays a crucial role in calcium (Fraser, 1970 and Boyle, 1972) and phosphorus homeostasis (De Luca, 1974). The control of production of the active metabolite, 1,25(OH)₂D₃, is critical. The accepted view is that 1,25(OH)₂D₃ is produced in accordance with strict physiological signals, in response to calcium and phosphorus demand. The regulation occurs at the 1 α hydroxylation of the 25(OH)D₃ i.e. the enzyme 25 hydroxyvitamin D₃-1-hydroxylase (1 α OHase). The enzymatic activity is affected by changes in serum calcium (Boyle, 1971), serum phosphate (Tanaka, 1973), parathyroid hormone (PTH) (Garabedian, 1972), calcitonin, cyclic AMP, vitamin D metabolites, and other associated factors including pH, steroid hormones and bisphosphonates (Norman, 1979). The complex interplay of hormones and other humoral factors are shown in figure 1.2.2.. The primary stimuli of the renal 1 α OHase were PTH, low serum calcium and low serum phosphate. Low serum phosphate appears to act on the kidney possibly through changes in the ionic environment of the kidney mitochondria and thereby promoting the 1 α hydroxylation of 25(OH)D₃. Parathyroid hormone secretion is promoted by low serum ionised calcium and the trophic effect of PTH on 25(OH)D₃-1-hydroxylase leads to increased levels of 1,25(OH)₂D₃. Having raised the levels of 1,25(OH)₂D₃ in the blood, the active metabolite acts on bone and intestine to mobilise and absorb calcium and phosphate. The loop was closed and the original hypophosphataemic and hypocalcaemic provocations are corrected by this classic endocrine feedback system. However, the system is generally challenged by one or other of the conditions but not usually both together. As 1,25(OH)₂D₃ increases the concentration of both ions, one can envisage a situation where there can be excess phosphate in the response to a hypocalcaemic challenge. During the compensation of the low serum calcium the excess phosphate is eliminated in the urine as a direct result of PTH inhibiting the renal reabsorption of phosphate. In contrast, excess calcium mobilised in response to hypophosphatemia is corrected by a direct inhibitory action of

Calcium and Phosphorus Homeostasis

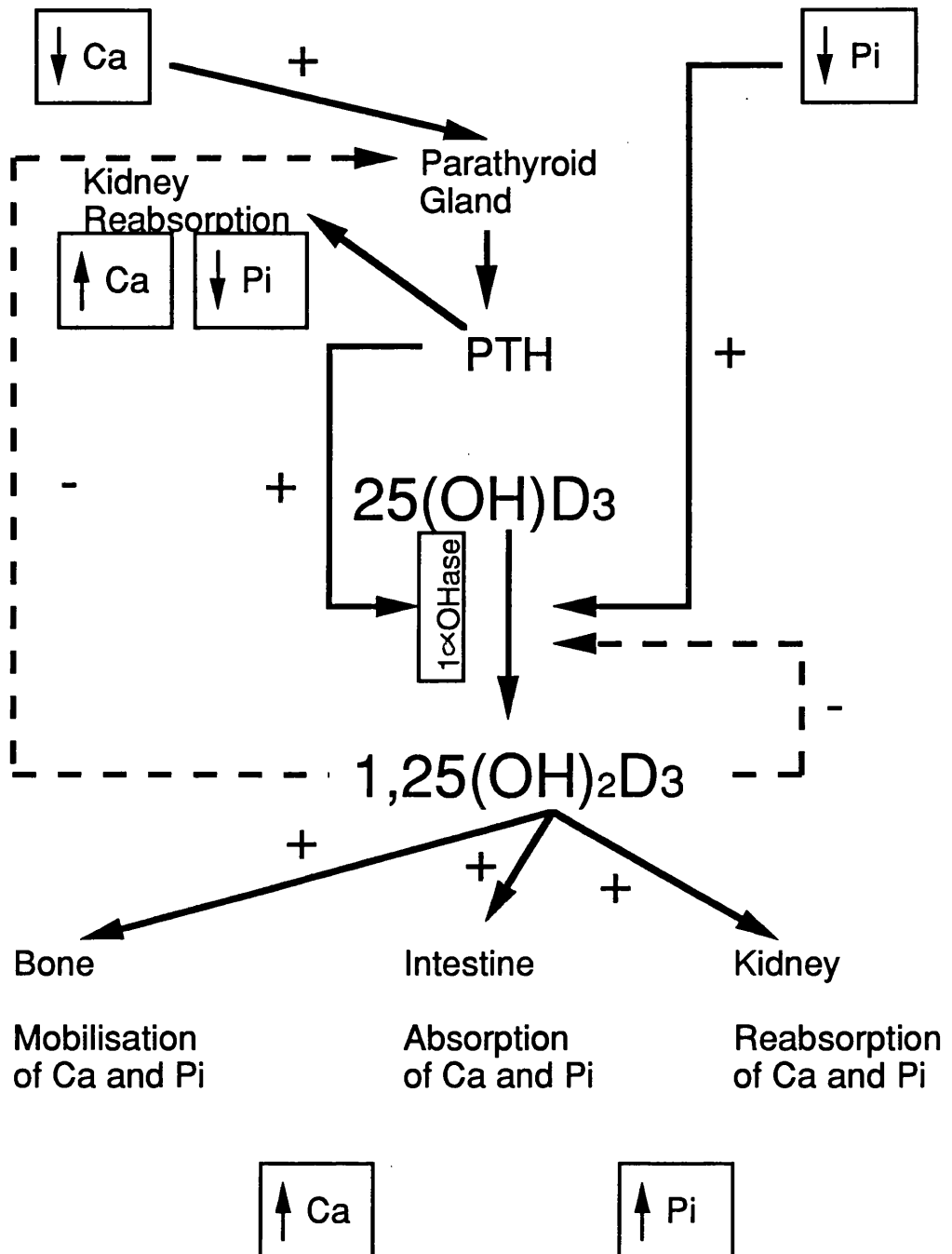


FIGURE 1.2.2.

Calcium and Phosphorus homeostasis : A flow chart of Calcium and Phosphorus homeostasis. The chart demonstrates the interaction between the vitamin D endocrine system and the parathyroid hormone system. The feedback control mechanisms are also demonstrated.

1,25(OH)₂D₃ on the parathyroid gland. In this situation, PTH is suppressed and therefore is not able to cause enhanced renal calcium reabsorption. The homeostasis system works because PTH participates in one of the loops, the calcium loop and in addition PTH has opposite effects on the renal handling of both calcium and phosphate ions.

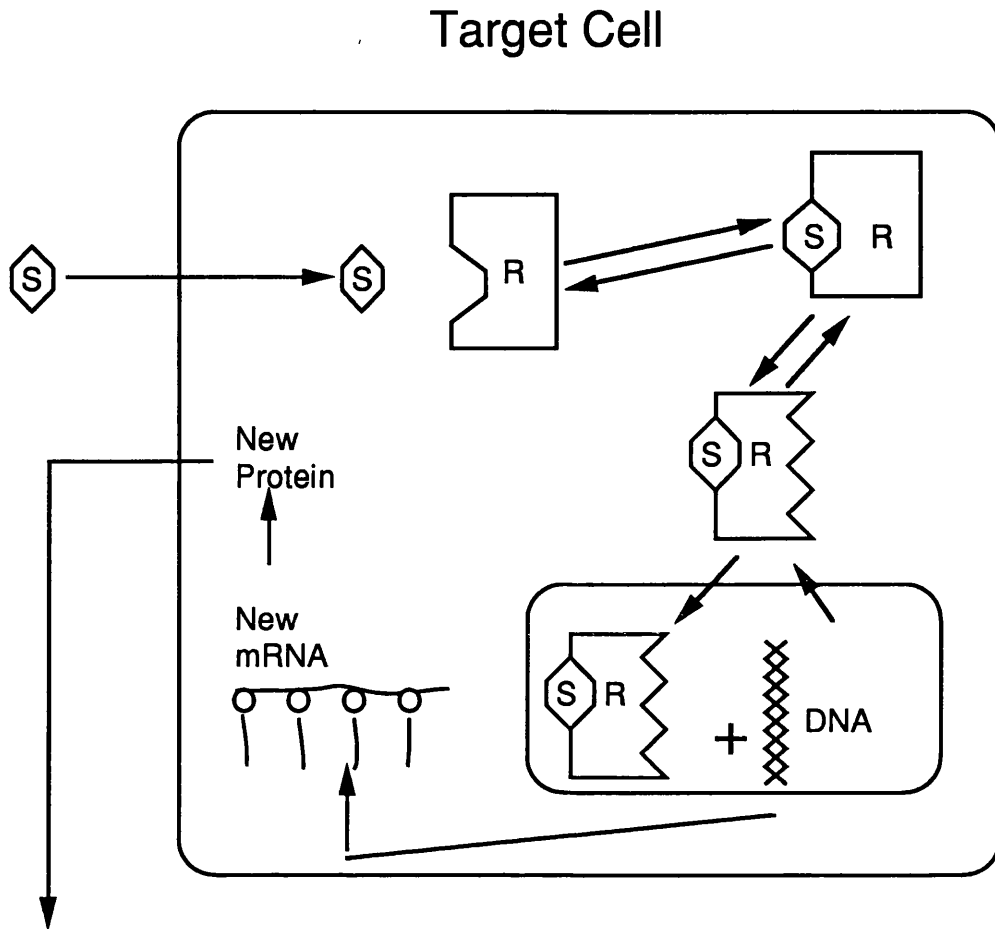
It is possible that 1,25(OH)₂D₃ can regulate its own biosynthesis by acting as a feedback regulator on either the parathyroid gland or the kidney or both (Norman, 1980). The presence of 1,25(OH)₂D₃ can induce the formation of the renal 24-hydroxylase (24(OH)ase) which uses up excess 25(OH)D₃ and therefore establishes a negative feedback loop. Both 1,25(OH)₂D₃ and 24,25(OH)₂D₃ may modulate PTH secretion through a short feedback loop. The consensus of opinion favours the long loop feedback mechanism that occurs between the product, ionised calcium in blood, and the parathyroid gland as the main regulator of the 1 α OHase. The short loop could play an important role as it has been established that parathyroid chief cells possess receptors for 1,25(OH)₂D₃ (both cytosolic and nuclear). Infusion of both 1,25(OH)₂D₃ and 24,25(OH)₂D₃ directly into the gland results in an inhibition of PTH secretion whereas conversely infusion of 1 α hydroxyvitamin D₃ into blood does not result in PTH inhibition (unpublished observation S. Gallacher). This modulation of PTH by the 1,25(OH)₂D₃ is reminiscent of classic steroid hormones such as glucocorticoids or oestrogens that feedback directly on to the hypothalamus and pituitary to inhibit the secretion of adenocorticotrophic hormone (ACTH) and follicle stimulating hormone (FSH). Growth hormone, prolactin, oestrogens and possibly glucocorticoids may also modulate 1,25(OH)₂D₃.

1.2.5. Steroid Hormone Mode of Action.

The mode of action of $1,25(\text{OH})_2\text{D}_3$ on target cells is analogous to that of classical steroid hormones. The time scale of events is in keeping with that of accepted models. Administration of $1,25(\text{OH})_2\text{D}_3$ is followed by a latent period of approximately two hours before a detectable physiological response was noted. This lag was sufficiently long to allow induction of proteins but at the same time short enough to confirm $1,25(\text{OH})_2\text{D}_3$ as the mediator of the response.

In simplistic terms the two step model of action of steroid hormones is represented in figure 1.2.3.. $1,25(\text{OH})_2\text{D}_3$ (S) binds to a cytoplasmic high affinity receptor (R). The hormone-receptor complex undergoes a conformational change. The hormone-receptor complex then migrates to the nucleus, where it binds to the specific acceptor site. At this point there was a resultant stimulation of template activity, which results in the production of messenger RNA (mRNA). Messenger RNA then migrates into the cytoplasm and is translated into new proteins which facilitate biological response.

Steroid Hormone : mode of action



Biological
Response

FIGURE 1.2.3.

The mode of action of the steroid hormone is presented in this figure. The steroid hormone (S) interacts with its target tissue by binding to a specific protein receptor localised in the cytoplasmic portion of the cell. The steroid - hormone complex (SR) undergoes a conformational change prior to entry into the nucleus. The SR complex then interacts with the DNA to initiate transcription to produce new mRNAs which are subsequently translated to give new proteins and hence a biological response.

1.3 THE IMMUNE SYSTEM.

1.3.1. Early beginnings.

The Latin words *imunitas* and *immunis* were used to describe those that were exempt from service (military) to the state. From this point the words developed connotations with the ancient observation that people who had been smitten with certain diseases and subsequently recovered seldom contracted these diseases again i.e. were exempt hence the term immunity (Silverstein, 1989). Over the last two centuries it has been recognised that the immune system comprises organs, cells and molecules whose function was to recognise and eliminate foreign bodies. The system was orchestrated by a number of specialised blood cells. Recently it has become apparent that the system was regulated by hormones in much the same way as other systems within the body. These immune system hormones have been termed interleukins - a communication link between leukocytes.

1.3.2. Cells of the immune system.

The cells involved in the immune system (figure 1.3.1.) can be categorised as follows ;

Monocytes.

Monocytes are cells derived from a myeloid stem cell precursor. The cells are formed in the bone marrow and circulate in blood, to tissues where they mature into macrophages (M ϕ). They function as scavengers although their prime function is presentation of antigen.

Lymphocytes.

There are two broad sub-types of lymphocytes and they derive their names from the sites where the immature cells differentiate to form mature cells. There are the T-lymphocytes that are thymus derived and the B-lymphocytes derived from the 'bursa equivalent' in man. Although B and T lymphocytes are morphologically similar they perform distinct functions. T-lymphocytes (T-cells) participate in cell mediated reactions and also help regulate antibody production by B-lymphocytes (B-cells). B-lymphocytes mature into plasma cells that produce antibody.

T-cells do not produce antibody but they do express specific antigen receptors that resemble antibody. These receptors bind antigen presented by macrophages in the context of major histocompatibility complex molecules. The binding of antigen results in a series of reactions culminating in the secretion of molecules that mediate their immune function. On the basis of the molecules secreted and the expression of surface receptors T-cells can be further divided into helper/inducer T-cells (T_H, CD4+ve) and cytotoxic/suppressor T-cells (T_S, CD8+ve). T_H perform their function by secreting interleukins whereas T_S make direct contact with infected cells, secrete toxic molecules, kill the cells and the microbes therein.

Cells involved in immunity

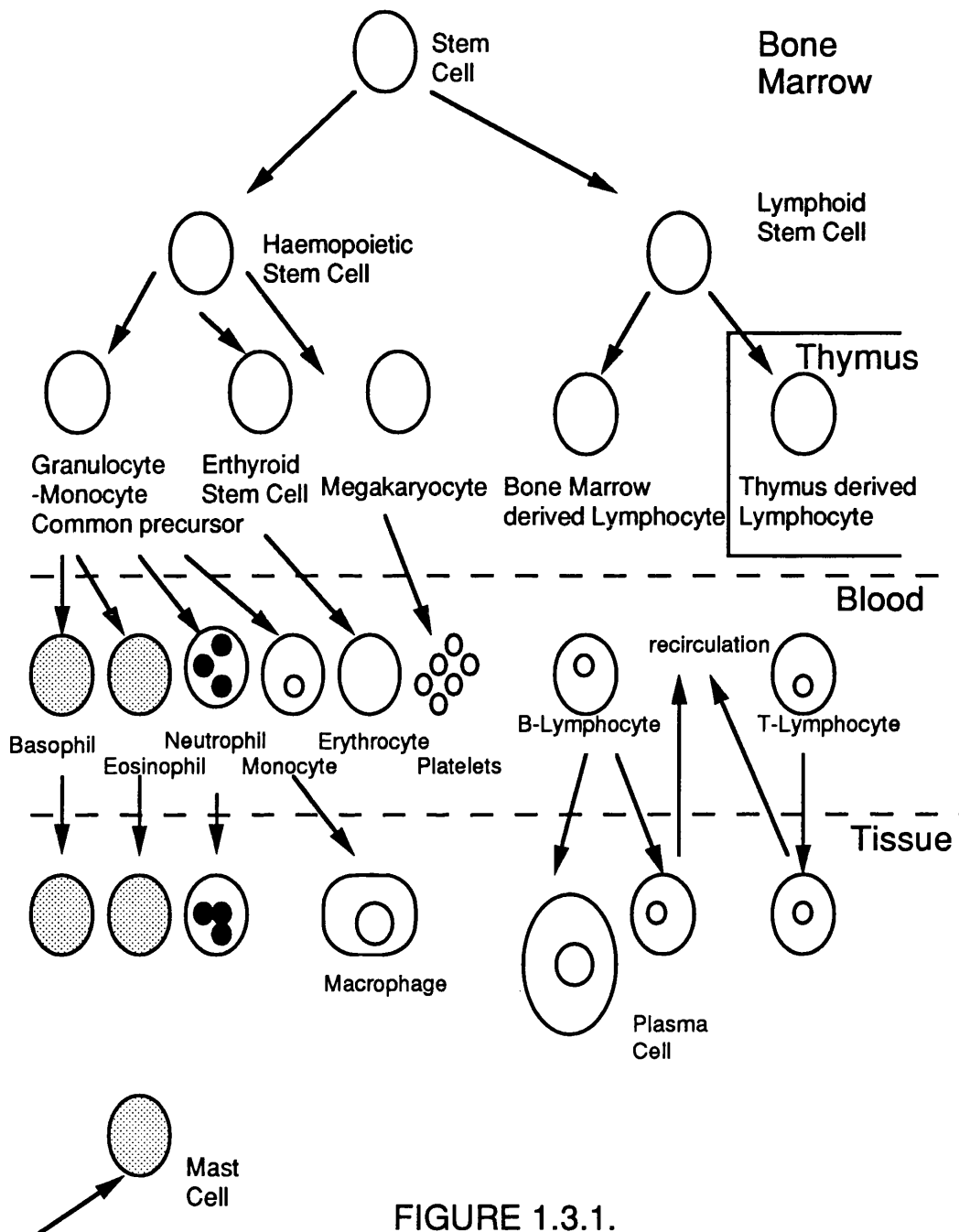


FIGURE 1.3.1.

The haemopoietic system : Cells involved in immunity. The diagram represents the cells involved in immunity and their development to immunocompetent cells at both the blood level and at the level of tissues. The differentiation pathway of the various mature cells are presented. All the cells are derived from a common stem cell.

Granulocytes.

There are three subtypes of granulocytes; neutrophils, eosinophils and basophils. These cells are also produced in the bone marrow. Neutrophils perform phagocytosis of microorganisms. Eosinophils are also capable of phagocytosis possibly of larger organisms. Basophils are like mast cells in that they contain powerful chemicals such as histamine and they may be involved in the allergic response.

1.3.3. Properties of the immune system.

The immune system has evolved the ability to sense and respond to foreign molecules. Foreign molecules can take the form of microorganisms such as viruses, bacteria, fungi and parasites whose recognition as foreign molecules is mediated by T and B lymphocytes. The immune system possesses three novel characteristics. Firstly, reaction to a foreign molecule (antigen) is both highly specific and at the same time diverse. This is achieved by having unique lymphocytes that can respond to unique antigens and is explained by the clonal selection theory (Burnet, 1958). Basically this infers that for every possible antigen there is a specific lymphocyte that can recognise this antigen. Exposure to an antigen leads to clonal expansion of a specific lymphocyte and hence an immune response. A second characteristic, requires that the immune system has the ability to distinguish between self and nonself and this ensures that the body does not turn on itself. This attribute is achieved by the major histocompatibility complex located on chromosome six. In a small section of chromosome 6 there are four gene loci (D,B,C,A) on each strand of the chromosome, and these eight genes (four loci on each chromosome strand) code for the protein that make up the self antigen. These self antigens are expressed on every cell that make up the genetic individual known as self. Finally, once the system is exposed to a particular antigen, the response time and the force with which it responds to subsequent exposure is heightened. This final feature implies that the immune system has a memory. Again this can be related to clonal selection in that the T-cells activated in the initial exposure are still viable and are actively involved in surveillance hence there are more T-cells with a specific antigen binding protein available to meet the challenge.

1.3.4. Interleukins - The hormones of the immune system.

Within the past ten years, it has become apparent that the immune response is controlled by a group of proteins that have been termed interleukins. Having defined interleukins it is perhaps advantageous to define the other terms commonly used in this research field. Lymphokines are defined as soluble mediators produced by lymphocytes whereas cytokines are mediators produced by cells. At present there are ten recognised interleukins (table 1.3.1.) but this list is likely to expand. Each interleukin is unique although it is possible that some of their activities may overlap. Characterisation of the interleukins has enabled immunologists to confirm that these proteins act on the immune system in much the same way as the classical hormones and their receptors act on the endocrine system.

Interleukin-2 (IL-2) was the first hormone of the immune system to be discovered and characterised (Gillis, 1978) and has a crucial role to play in the generation of an effective immune response. If peripheral blood mononuclear cells were challenged with a mitogen (phytohemagglutinin (PHA)) then a supernatant was obtained that can support the continual growth of T-cells (Morgan, 1976). This supernatant contains T-cell growth Factor (TCGF) which is now known as IL-2. In the context of an immune response, IL-2 mediates the clonal expansion of antigen activated T-cells.

Interleukin-1 (IL-1) is produced by many cell types including fibroblasts, endothelial cells, keratinocytes and smooth muscle. However by far the most important source of IL-1 is the monocyte/macrophage (Mizel, 1989). IL-1 has a broad range of functions to perform in the immune response. These include regulation of various aspects of T and B lymphocyte development and function, induction of interleukins and interleukin receptor synthesis and expression, and lymphocyte proliferation (Mizel, 1988; Dinarello, 1984; Shirakawa, 1986). Clearly IL-1 has an important role to play in the immune system, but, it is also involved in bone remodelling.

The Interleukin Family

Interleukin	Previous Names
IL-1	Endogenous Pyrogen (EP) Lymphocyte Activating Factor (LAF) Leukocyte Endogenous Mediator (LEM) Catabolin Mononuclear Cell Factor (MCF)
IL-2	T-Cell Growth Factor (TCGF) T-Cell Maturation/Stimulating Factor (TMF/TSF) Killer Helper Factor (KHF) T-Cell Replacing Factor (TRF)
IL-3	Multiple Colony Stimulating Factor (Multi - CSF)
IL-4	B-Cell Factor I (BCGFI)
IL-5	B-Cell Factor II (BCGFII) Eosinophil Differentiation Factor (EDF)
IL-6	Hybridoma Growth Factor (HGF) Interferon β_2 (IFN β_2) B-Cell Stimulatory Growth Factor (BSF-2) 26 kDa protein Hybridoma/Plasma-cytoma Growth Factor (H/PGF) B-Cell Differentiation Factor (BCDF) B-Cell Stimulatory Factor p2 (BSFp2)
IL-7	B-Cell Growth Factor (BCGF)
IL-8	Neutrophil-Activating Peptide-1 (NAP1)
IL-9	Mast Cell Growth Factor P40
IL-10	Th1 Cytokine Synthesis Inhibitor

TABLE 1.3.1.

The Interleukin Family : The table presents the defined interleukins and their previous names giving a strong indication as to their function. The previous name refers to how these factors have been described in the scientific literature.

Interleukin-6 (IL-6) is an immune hormone that has a broad range of cell targets. Like IL-1, IL-6 is produced by many cell types including fibroblasts, monocyte/macrophages, endothelial cells, T and B cells, mesangial cells, keratinocytes and a number of tumour types (Mizel, 1989). IL-6 production is stimulated by IL-1, tumour necrosis factor α (TNF α), platelet derived growth factor (PDGF), virus infection, double stranded RNA and cyclic AMP. In the immune response IL-6 influences the growth and differentiation of T and B lymphocytes. IL-6 is involved in the conversion of T-cells into an IL-2 responsive state. IL-1 and IL-6 can be seen to be strongly synergistic in the induction of T-cell proliferation.

Although IL-1, IL-2 and IL-6 have been described above it is equally important to realise that the other interleukins also have important roles to play in an immune response.

1.4. VITAMIN D AND THE IMMUNE SYSTEM.

1.4.1 Vitamin D - Not just a calcium homoeostatic steroid hormone.

The role of vitamin D on calcium homeostasis is well documented. The active form of the steroid hormone, $1,25(\text{OH})_2\text{D}_3$, acts on the target tissues, intestine, bone and kidney, to mobilise calcium. As expected these so called target tissues were found to be rich in vitamin D receptors (VDR). However, as techniques for measuring VDR's improved it became apparent that many other cell types expressed these receptors. This led to an appreciation of the universal scope of 'the vitamin D endocrine system'.

It has long been suspected that there was a link between the vitamin D endocrine system and immune responses. Urist and McLean (Urist, 1956) observed that in rats on a low calcium / vitamin D diet there was a several hundredfold increase in their mast cells within the bone marrow. This was clearly evidence for involvement of vitamin D in some aspects of haematopoiesis. Clinical observations in rickets supported this theory. The rachitic patient exhibits anaemia, myelofibrosis and extramedullary haematopoiesis, and these are corrected by vitamin D therapy (Yetgin, 1982). Also of note was the observation that children with rickets were more susceptible to infections (Stroder, 1975). Investigation of these children revealed that they exhibit sub normal phagocytosis and reduced neutrophil motility (Yetgin, 1982).

1.4.2. Vitamin D receptor distribution - Universal ?

A detailed search for vitamin D receptors (VDR's) in various tissues revealed widespread distribution. Nearly every vertebrate cell type possessed these high affinity VDR's. Of particular interest was the discovery of VDR's in the cells of the immune system. The demonstration that monocytes and activated, but not resting, lymphocytes possess VDR's led to the hypothesis that $1,25(\text{OH})_2\text{D}_3$ may play a role in immunoregulation (Provvedini, 1983; Bhalla, 1983). VDR's were not restricted to

mature cells and are found for example to be present in mitotically active thymoblasts (Ravid, 1984).

1.4.3. Vitamin D - A differentiation factor.

The widespread distribution of VDR's and in particular their presence on some tumour cells suggested a role for vitamin D in cell differentiation. In 1981, it was reported that $1,25(\text{OH})_2\text{D}_3$ induced the differentiation of both a murine (Abe, 1981) and a human myeloid leukemic cell line (Miyaura, 1981), and that this occurred within the normal physiological range for this metabolite. The two cell lines involved were the mouse myeloid leukaemia cell line (M1) and the human promyelocytic leukaemia cell line (HL60). Interestingly the differentiated product was different in each case : M1 were differentiated down to macrophages whereas HL60 were differentiated to granulocytes. Further evidence for a role in myeloid differentiation comes from work on a human monocytic cell line, U937. This cell line was also induced to differentiate by $1,25(\text{OH})_2\text{D}_3$ to macrophages (Amento, 1984). Although it was clear that $1,25(\text{OH})_2\text{D}_3$ had anti-tumour activity *in vitro* it was not until 1983 that an anti-tumour effect was demonstrated *in vivo*. Work carried out on nude mice, by a group in Japan, revealed that the survival time of mice inoculated with M1 cells was prolonged by administration of either $1,25(\text{OH})_2\text{D}_3$ or its synthetic analogue, 1α hydroxyvitamin D₃ (1α OH D₃) (Honma, 1983).

1.4.4. Extra-renal synthesis of 1,25(OH)₂D₃.

It was now well established that the principle site for the synthesis of 1,25(OH)₂D₃ was the kidney (Fraser, 1970). Until recently, the only other source of 1 α hydroxylase activity was in the placenta (Gray, 1979). However, it is now apparent that more widespread extra-renal synthesis exists and may be an important factor in the context of the local environment. A major new insight was provided in 1981 with a case report describing an anephric individual with hypercalcemia and elevated 1,25(OH)₂D₃ who was diagnosed to have sarcoidosis (Barbour, 1981). This disorder is granulomatous in nature and of unknown etiology. Following this observation it was found that sarcoid lymph node homogenates and alveolar macrophages possessed 1 α hydroxylase activity, and that this may account for the raised 1,25(OH)₂D₃ and consequently the hypercalcemia (Adams, 1983; Mason, 1984). The substrate specificity and enzyme affinity for 25(OH)D₃ of this 1 α hydroxylase was indistinguishable from those reported for the renal enzyme (Adams, 1985). Significantly, in patients with sarcoidosis, the degree of hypercalcemia correlates with the V_{max} of alveolar macrophage 1 α hydroxylase activity measured *in vitro* (Adams, 1985). As a consequence monocytes and macrophages from normal individuals were studied to see if they possessed 1 α OHase activity. Both 1 α OHase and 24 hydroxylase activity could be demonstrated in monocytes/macrophages cultured in the presence of either lipopolysaccharide (LPS) or interferon- γ (IFN- γ) (Koeffler, 1985; Reichel, 1987). Clinical data suggests that 1 α OHase can be induced by various other stimuli such as infection and foreign body reactions (Kozeny, 1984; Felsenfeld, 1986).

1.4.5. 1,25(OH)₂D₃ - A novel immunoregulatory hormone.

As a consequence of the distribution of vitamin D receptors and the extra-renal synthesis of 1,25(OH)₂D₃ it became obvious that 1,25(OH)₂D₃ must have a wider biological role than was first envisaged. In 1984, Tsoukas et al presented data that demonstrated 1,25(OH)₂D₃ at picomolar concentrations inhibited cell proliferation and interleukin-2 activity (Tsoukas, 1984). They discussed the potential mechanism by which 1,25(OH)₂D₃ mediates this effect. Several possibilities were discussed;

- (1) 1,25(OH)₂D₃ may act directly on the synthesis of IL-2 or on the secretion of IL-2 or both.
- (2) 1,25(OH)₂D₃ may increase consumption of IL-2 by increasing production of the receptor for IL-2 either through transcription or post translational mechanisms.
- (3) 1,25(OH)₂D₃ may inhibit IL-2 by acting on the differentiation pathway of suppressor lymphocytes.

It is these possibilities that are addressed in this thesis. Evidence from Lemire et al suggested that 1,25(OH)₂D₃ inhibited both IgG and IgM production. They also confirmed the effect on cell proliferation (Lemire, 1984). Taken together these findings provide a strong case for a role for 1,25(OH)₂D₃ in the regulation of the immune response.

The precise nature of this role of $1,25(\text{OH})_2\text{D}_3$ in the immune system remains to be clarified. It appears that $1,25(\text{OH})_2\text{D}_3$ inhibits RNA synthesis between early and late G1 in the cell cycle (Manolagas, 1986). It also transpires that $1,25(\text{OH})_2\text{D}_3$ appears to mediate its antiproliferative effect through both IL-2 dependent and IL-2 independent mechanisms (Rigby, 1985). Furthermore, it has been suggested that $1,25(\text{OH})_2\text{D}_3$ may inhibit the mRNA for IL-2 but have little effect on IL-2 receptor (IL-2R) (Rigby, 1988). $1,25(\text{OH})_2\text{D}_3$ appears indirectly to regulate B-cell growth and differentiation through its effects on T-cells (Lemire, 1985), but effects on B-cells have to be further characterised.

The influence of $1,25(\text{OH})_2\text{D}_3$ on the immune system is not restricted to cells of lymphoid origin. As discussed previously monocyte/macrophage possess vitamin D receptors and are capable of producing $1,25(\text{OH})_2\text{D}_3$. $1,25(\text{OH})_2\text{D}_3$ appears to promote the fusion of macrophages and augment Fc receptor expression and cytotoxic function (Abe, 1984). The production of $1,25(\text{OH})_2\text{D}_3$ by monocytes may be of importance in the local environment, where it can induce heat shock proteins thereby protecting monocytes in the situation of thermal stress brought on by tissue inflammation (Polla, 1986). It has also been reported that $1,25(\text{OH})_2\text{D}_3$ increased IL-1, PGE_2 and TNF production by monocytes.

1.4.6. $1,25(\text{OH})_2\text{D}_3$: The future.

$1,25(\text{OH})_2\text{D}_3$ may play a unique role functioning as both an endocrine and a paracrine hormone, in distinct biological systems. These roles may both be mediated via $1,25(\text{OH})_2\text{D}_3$'s immunomodulatory activity. In the context of mineral metabolism, $1,25(\text{OH})_2\text{D}_3$ treated monocytes produce increased amounts of IL-1, TNF and PGE_2 all of which can induce bone resorption. Also $1,25(\text{OH})_2\text{D}_3$ can block IFN- γ which has the effect of mobilizing calcium from bone (Rigby, 1988). Taken together the overall effect of $1,25(\text{OH})_2\text{D}_3$ in the immune system on bone skeleton is bone resorption and increased calcium mobilisation.

1,25(OH)₂D₃ and in particular its synthetic analogue 1 α OH D₃ have emerged as possible therapeutic agents in immunoregulatory disorders. However, treatment of myeloproliferative disorders with 1,25(OH)₂D₃ has been far from convincing (Koeffler, 1985). On the positive side, some low grade lymphomas responded to treatment (Cunningham, 1985). The most convincing benefits have been seen in the treatment of psoriasis with 1,25(OH)₂D₃, a skin disease characterised by disturbed epidermal cell proliferation and altered cell mediated immune responses (Morimoto, 1986). The next step forward would be the development of 1,25(OH)₂D₃ analogs without the problems associated with hypercalcemia. These analogs if available would be important immunosuppressors that do not have the toxicity of present regimes such as glucocorticoids and cyclosporin A therapy.

CHAPTER 2.

MATERIALS AND METHODS.

2.1 NORMAL VOLUNTEERS.

Peripheral blood mononuclear cells (PBMC) were obtained from a pool of normal healthy volunteers. This pool included staff from the laboratory, wards and clinics. In general wherever possible the work was carried out on males.

Blood was obtained by venepuncture and collected into a universal containing 300 i.units of preservative-free heparin (final concentration in blood; 20 i.units/ml, Leo Laboratories).

2.2 CELL SEPARATION.

2.2.1. Peripheral Blood Mononuclear Cells (PBMC).

PBMC's were separated by density gradient centrifugation using a modification of the method originally described by Bøyum (Bøyum, 1964, 1968) (see figure 2.2.1). Heparinised blood was diluted 1:1 with Eagles minimum essential medium (MEM) and layered on to an equal volume of lymphoprep™ (Nyegaard and Co., Oslo, Norway) in 15 mls. plastic conical tubes (diameter 12mm., Starstedt). Following centrifugation at 500 x g for 30 minutes at room temperature, the mononuclear cell bands at the lymphoprep / plasma interface were removed using a Pasteur pipette (see figure 2.2.2). The band from two tubes were pooled and washed three times in MEM by centrifugation at 400 x g for 10 minutes at 4 °C, and the cell pellet resuspended in 1 ml cell culture medium. The cell number was assessed in 10 µl aliquot of cells stained with 90 µl of white cell staining fluid (2% glacial acetic acid, a few grains of crystal violet in phosphate buffered saline (PBS)) and counted in a standard haemocytometer (Improved Neubauer). The cell suspension was stored on ice until required.

Peripheral Blood Mononuclear Cell Separation

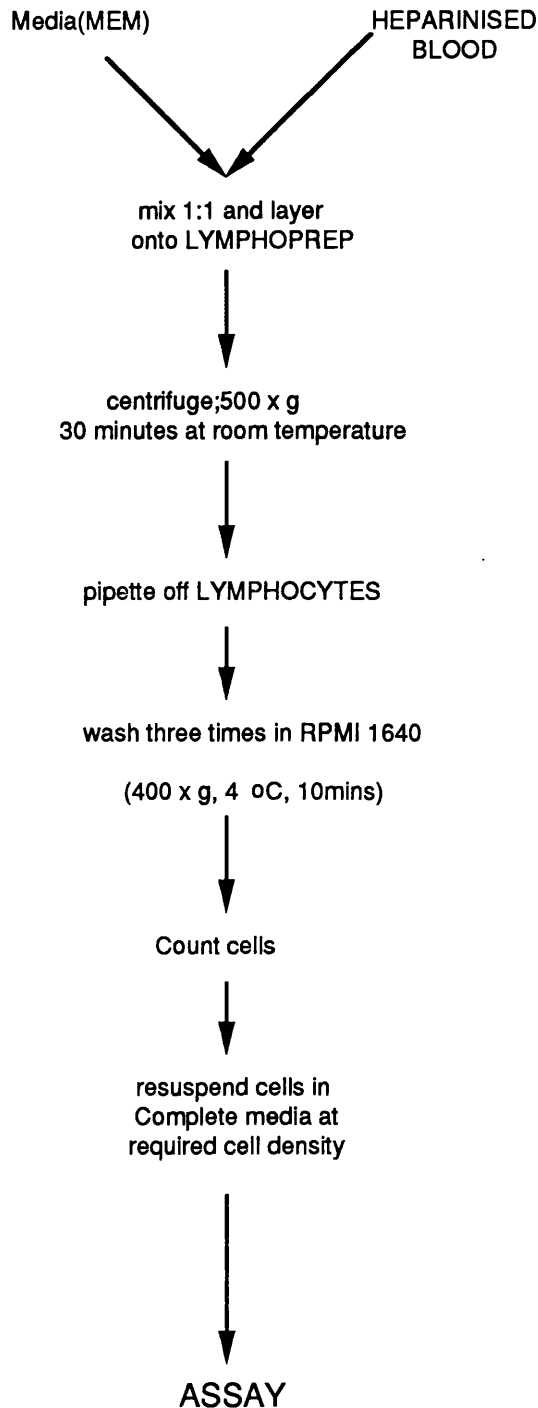


FIGURE 2.2.1

The protocol for separating PBMC's by density gradient centrifugation over LymphoprepTM as outlined by Boyum.

Peripheral Blood Mononuclear Cells Separation -
layered on Lymphoprep

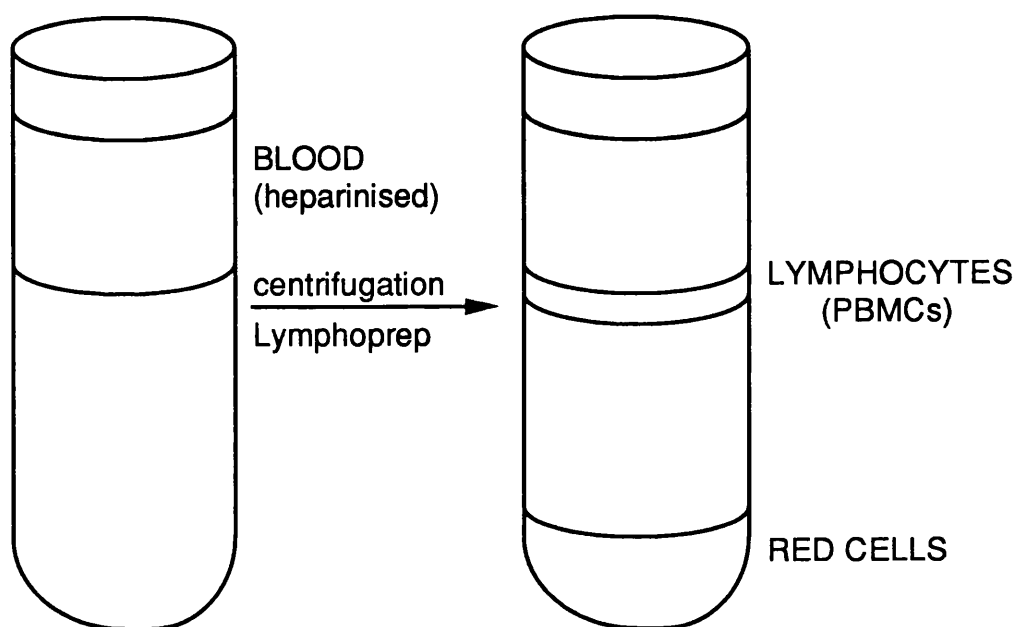


FIGURE 2.2.2.

Diagrammatic representation of the procedure for separating PBMC's over lymphoprep TM. The situation before centrifugation is represented on the left hand side and the end result of centrifugation is represented on the right hand side.

2.2.2 Lymphocyte sorting - CD4+ve and CD8+ve.

The technique employed to separate out the required subsets from a mixed population of PBMC's was a commercially available immunomagnetic separation system (IMS). This system was preferred to the alternative panning technique which was initially used. The panning technique involved absorbing monoclonal antibodies against the appropriate antigenic subset onto polystyrene plates. The required population could be attained by either a positive or a negative selection procedure. Although the subsets could be pulled out using this method the yield was not satisfactory and consequently the commercial IMS was employed. The principles of the IMS were quite straightforward ; one has a uniform sized particle that has a magnetised core and is coated with a specific monoclonal antibody. These particles are incubated with a cell population, the coating antibody recognises a specific cell surface antigen and binds to the cell. A magnetic field is applied and the cells that have particles attached to them are attracted to the magnet, thereby separating this cell population from the rest.

The products Dynabeads ® M450 CD4, T_{helper/inducer} and Dynabeads ® M450 CD8, T_{cytotoxic/suppressor} were obtained from Dynal (U.K.) Ltd., 24 - 26 Grove street, New Ferry, Wirral L62 5 AZ. Each particle is coated with a mouse monoclonal that recognises either a single chain 55 - 65 kd cell membrane glycoprotein in the case of CD4 or a double chain 32 - 43 kd cell membrane glycoprotein for CD8. Both the CD4 and the CD8 antigens were designated such at the First International Workshop and Conference on Human Leukocyte differentiation antigens.

PBMC's were separated out as previously described in section 2.2.1.. After the final wash the PBMC's were resuspended in PBS + 1% FCS at a cell density of 3×10^6 cells/ml. In order to have approximately the same number of target cells for the Dynabeads[®] M450 CD4 and the Dynabeads[®] M450 CD8, the cell suspension was split in a ratio of 1:2 and added to 15mls. polypropylene tubes (Bibby, U.K.). The Dynabeads[®] were washed three times in PBS + 1% FCS, the particles retained by the Dynal[®] Magnetic Particle Concentrator (MPC), the magnetic field was applied for two minutes and the wash fluid was pipetted off. Having been washed the magnetic beads were resuspended in their original volume of PBS + 1% FCS. Manufacturers recommendations for gradient cells were that there should be a ratio of 3:1 (beads:cells). On the basis of this the beads were added to the appropriate tubes and incubated at 4 °C for 10 - 20 minutes for CD8 and for 30 minutes for CD4. The incubation was preformed on a rotating drum. The rosetted cells were separated by placing the polypropylene tube in the MPC for 2 - 3 minutes. The supernatant was aspirated out and can be kept to represent a depleted population, the rosetted cells are located on the walls of the tube held there by the magnetic field. The rosetted cell population is then washed a further five times with PBS + 1% FCS. The cells were resuspended in culture fluid, RPMI 1640 + 20% FCS, and counted. The resuspended cells were stored on ice until required.

2.3. CELL LINES.

2.3.1. HT-2.

The HT-2 cell line was obtained from the Cetus Corporation, 1400 Fifty-third Street, Emeryville, California 94608, U.S.A.. The cells were maintained at a cell density of 4×10^4 cells/ml in RPMI 1640 supplemented with 10% FCS, 20 units/ml recombinant IL-2 (rIL-2) (BCL, Boehringer Mannheim House, Bell lane, Lewes, East Sussex BN7 1LG) and 2×10^{-5} M 2- β -mercaptoethanol (20 mM stock made up in PBS). The cells were split and fed every 48 hours. HT-2 cells are derived from murine T helper cells, more specifically from the strain Balb/c (Watson, 1979). The cells are dependent on either murine, rat or human IL-2.

2.3.2. LBRM TG6.

The LBRM TG6 cell line was also obtained from the Cetus Corporation. The cells were maintained at a cell density of 10^5 cells/ml in Iscove's modification of Dulbecco's MEM (IDMEM) + 10% FCS. LBRM TG6 is a subclone of LBRM 33 (Larrick, 1985). The cells are resistant to both 5×10^{-5} M 8 azaguanine and 2×10^{-5} M 6 thioguanine, and the cells died when cultured in conventional HAT and in hypoxanthine/azaserine supplemented medium. LBRM TG6 produce IL-2 only in the presence of lectin and IL-1.

2.3.3. B9.

B9 cells were a gift from Dr Lucien Aarden, Central Laboratory of the Netherlands, Red Cross Blood Transfusion Service. The B9 were maintained at a cell density of 4×10^4 cells/ml in Iscoves Modified Dulbecco's MEM, 5% FCS, 5×10^{-5} M mercaptoethanol, penicillin and streptomycin (Aarden, 1987). The cells were further supplemented with 8 units/ml Hybridoma growth factor (HGF, Jansen). B9 may also be cultured in RPMI 1640.

2.4 MEDIA.

2.4.1. Rosewell Park Memorial Institute (RPMI) 1640.

RPMI 1640 (10 x) was obtained from Gibco, BRL and the culture media was prepared as per manufacturers instructions. Distilled/deionised water (ddH₂O) was autoclaved for 20 minutes at a temperature of 120 ° C and a pressure of 1.2 kg/cm². The ddH₂O was allowed to cool before the RPMI 1640 (10 x) was added, sodium bicarbonate (7.5%, Gibco, BRL) was added to give either a final concentration of 2 g/l in the case where it is to act as a CO₂ buffer as well as a nutrient or a final concentration of 0.85 g/l where N-[2-Hydroxyethyl]piperazine-N-[2ethanesulphonic] acid (HEPES) is the CO₂ buffer of choice. HEPES (1M, Gibco, BRL) was added to the media to give the concentration of choice (usually 10 mM). Finally the various supplements were added as detailed elsewhere in the text, these were added prior to use. The bottle of media is then adjusted to the correct pH (between pH 7.2 and pH 7.4) by addition of either HCl or Na OH.

2.4.2. Minimum Essential Medium with Hanks salts (MEM).

This media is a multi-purpose media that is used principally in this instance for washing cells (see section 2.2.1.). The media was prepared in a similar fashion to RPMI 1640. Distilled/deionised water was autoclaved and Hanks Balanced Salt Solution (10 x) (HBSS, Gibco, BRL) was added to the water after it had returned to room temperature. Amino acids (50 x, Gibco, BRL), Vitamins (MEM , 100 x, Gibco, BRL) and 20 mM HEPES were added to the water. Sodium bicarbonate (7.5%) was added to give a final concentration of 0.375 g/l. Prior to use 2mM glutamine (200 mM, Gibco, BRL) and 50 units/ml/50 µg/ml penicillin/streptomycin ((100 x), Gibco, BRL) were added to the media. The pH of the media was then adjusted to between pH 7.2 and pH 7.4.

2.4.3. Iscoves Modified Dulbeccos Medium (IMDM).

IMDM (1x, Gibco, BRL) was obtained as prepared media that was almost ready to use. As with other media the glutamine (200 mM) and the Penicillin/streptomycin were added prior to use.

2.4.4. Glutamine.

Glutamine (200 mM) was added to the bottle of media immediately prior to use. The reason for this is to do with the half-life of glutamine. Glutamine has a half-life in the order of 19 days at 4 °C, so it is important to replenish the glutamine lost. A rough calculation indicated that after 7 days at 4 °C only 80% of the original glutamine would be intact. It is for this reason that glutamine was added every week to bottles of media in use for longer than a week to make up for this shortfall. Glutamine was an essential nutrient and energy source for the growing cell. Stock glutamine was for this reason stored at -20 °C.

2.5. CALCITROPHIC HORMONES.

Calcitrophic hormones are naturally occurring substances that act to regulate calcium. Five calcitrophic hormones were studied as a group: 1,25 dihydroxyvitamin D₃ (1,25(OH)₂D₃), 24,25 dihydroxyvitamin D₃ (24,25(OH)₂D₃), 25 hydroxyvitamin D₃ (25(OH)D₃), parathyroid hormone (PTH) and calcitonin (CT).

2.5.1. The vitamin D metabolites.

All three D metabolites, 1,25(OH)₂D₃, 24,25(OH)₂D₃ and 25(OH)D₃, were obtained as a gift from Hoffman La Roche. The D metabolites were stored as ethanolic solutions. The concentration of each of the stock solutions were assessed by spectrophotometry. A working stock of 10⁻⁵ M was set up for each of the metabolites in ethanol (Analar grade, BDH). Subsequently, each working stock underwent a ten fold dilution in ethanol to produce ranges that ran from 10⁻⁵ M to 10⁻¹¹ M.

2.5.2. Parathyroid Hormone.

Bovine (1-35) parathyroid hormone (b(1-35)PTH) was used as replacement for full length human PTH. The b(1-35)PTH fragment is biologically active in all human systems thus far studied. The b(1-35)PTH was purchased from the Sigma Chemical Company, U.K.. The b(1-35)PTH was dissolved in 10mM acetic acid/0.1% bovine serum albumin (BSA) to give a working solution of 10⁻⁵ M. The working stock was diluted in a ten-fold manner in acetic acid/0.1% BSA to produce a range from 10⁻⁵ M to 10⁻¹¹ M.

2.5.3. Calcitonin.

Salmon calcitonin (sCT) was used and was supplied by Rorer Pharmaceuticals, U.K.. The concentration of the vials supplied was calculated to be 7.8×10^{-6} M. The sCT was present as a saline acetate solution and the working solution was as it was supplied. The range was constructed by an initial dilution (7.8 fold) to give a 10^{-6} M followed by ten-fold dilutions to complete the range (10^{-6} M to 10^{-11} M).

2.6. SEX HORMONES.

Three sex hormones were studied as a group : oestrogen, progesterone and dihydrotestosterone (DHT). All these hormone preparations were active both *in vivo* and *in vitro*. These hormones function within the endocrine system to control reproduction. However, they have also been implicated in other physiological systems.

2.6.1. Oestrogen.

Oestrogen is a female sex hormone that exerts dominant control over the development of all the accessory sex organs and secondary sex characteristics. Oestrogen was obtained from the Sigma Chemical company, U.K.. Oestrogen was purchased as a lyophilised product and it was reconstituted in ethanol to give a working stock of 10^{-5} M.

2.6.2. Progesterone.

Progesterone is also a female sex hormone that is present in significant amounts during the luteal phase of the menstrual cycle. Progesterone was obtained from the Sigma Chemical Company, U.K.. The progesterone was purchased as a lyophilised product and reconstituted in ethanol to produce a working solution of 10^{-5} M.

2.6.3. Dihydrotestosterone.

Testosterone is the male sex hormone that is required among others for spermatogenesis. In man, virtually all the obvious masculine secondary sex characteristics are testosterone dependent. Dihydrotestosterone is the biologically active form of testosterone found in the body. DHT was obtained from the Sigma Chemical Company, U.K.. The DHT was purchased as a lyophilised product and reconstituted in ethanol to produce a working solution of 10^{-5} M.

2.7. LYMPHOCYTE ACTIVATION (MITOGENESIS).

Lymphocyte activation by mitogens provide a means of looking at immune function. The lymphocyte activation assay along with cytokine assays provide a baseline evaluation of immune function.

2.7.1. Cell Preparation.

Venous blood was added as aseptically as possible to a universal containing preservative-free heparin such that the final concentration was of the order of 20 units/ml (see section 2.1.). The heparinised blood was mixed in a ratio of 1:1 with MEM. Peripheral blood mononuclear cells are separated by density gradient centrifugation over Ficoll - Hypaque, washed 3 x in MEM and resuspended in 1 ml RPMI 1640 media (see section 2.2.1.). The cells were counted and resuspended at (a) 10^6 cells/ml and (b) 2×10^6 cells/ml in complete RPMI 1640 (without HEPES) supplemented with 20 % pooled human sera (PHS). Complete media consists of RPMI 1640 with Glutamine, pen/strep and sodium bicarbonate.

2.7.2. Mitogens.

Three distinct mitogens were used in the system, phytohaemagglutinin (PHA), concanavilin A (ConA) and pokeweed mitogen (PWM). Each new batch of mitogen was calibrated to check the optimum stimulating concentration (see section 3.3.).

Phytohaemagglutinin (PHA).

PHA is a lectin extracted from kidney beans (*phaseolus vulgaris*), it is a T_H cell mitogen. PHA (Sigma Chemical Company, U.K.(Cat No L-9132)) was resuspended in sterile deionised water to give a stock solution of 1000 µg/ml. PHA stock was aliquoted and stored at -20 °C. Prior to use an aliquot of stock PHA was further diluted in complete RPMI 1640 (without PHS) to give a working concentration of 10 µg/ml (a 100-fold dilution).

Concanavilin A (ConA).

ConA is extracted from jack beans (*conavalia ensiformis*), it is a T_S cell mitogen. ConA (Sigma Chemical Company, U.K.(Cat No C-2010)) was resuspended in sterile deionised water to give a stock solution of 5 mg/ml. ConA stock was aliquoted and stored at -20 °C. Before use an aliquot of the stock was further diluted in complete RPMI 1640 (without PHS) to produce a working stock of 100 µg/ml (a 50-fold dilution).

Pokeweed Mitogen (PWM).

PWM is extracted from pokeweed roots (*Phytolacca americana*), it is a T-cell dependent B-cell mitogen i.e. it activates B-cells only in the presence of T-cells. PWM (Sigma Chemical Company, U.K.(Cat No L-9379)) was prepared at a concentration of 50 µg/ml in complete RPMI 1640 (without PHS), aliquoted and stored at -20 °C. Prior to use an aliquot of the stock was further diluted to give a working concentration of 0.2 µg/ml (a 250-fold dilution) in complete RPMI 1640 (without PHS).

2.7.3. Cell Culture.

The lymphocyte activation assay was set up in 96 well, U-shaped microtiter plates (Bibby Sterlin Ltd., U.K.) (see figure 2.7.1.). All cultures were set up in triplicate and in a final volume of 200 μ l. Each well contains 100 μ l of cells and 100 μ l of mitogen. In the case where PWM is the mitogen the stock cells were the cells resuspended at 2×10^6 cells/ml. The cultures were incubated at 37 °C in a humidified atmosphere of 5 % CO₂/Air for a total of 72 hours.

2.7.4. Labelling and Harvesting.

The most commonly used method of detecting lymphocyte activation is the incorporation of tritiated thymidine (³H-thy) into deoxyribonucleic acid (DNA), which correlates well with the number of lymphocytes in the S-phase of the cell cycle. The working stock contains 50 μ Ci ³H-thy (Cat No TRK 418, Amersham International p.l.c., U.K.(1mCi/ml)), 50 μ M 'cold' thymidine (stock 5mM) (Sigma Chemical Company, U.K.) and complete RPMI 1640 (without PHS). The equivalent of 1 μ Ci was added to each well (20 μ l) at 68 hours. The cultures were incubated in the presence of the label for further four hours.

After 72 hours the microtiter plates were harvested on a Titertek cell harvester (manufactured by Skatron for Flow Laboratories). The cell harvester transfers the cells from the wells to clearly defined discs on a glass-fibre filter mat (Skatron Ltd., U.K.) and unbound ³H-thy passes through the filter into the reservoir. The filter mats containing the cells were gently covered with two changes of 5 % trichloroacetic acid (TCA) (Sigma Chemical Company, U.K.) for ten minutes followed by two changes of methanol (A.R. Quality, James Burrough (FAD) Ltd., U.K.).

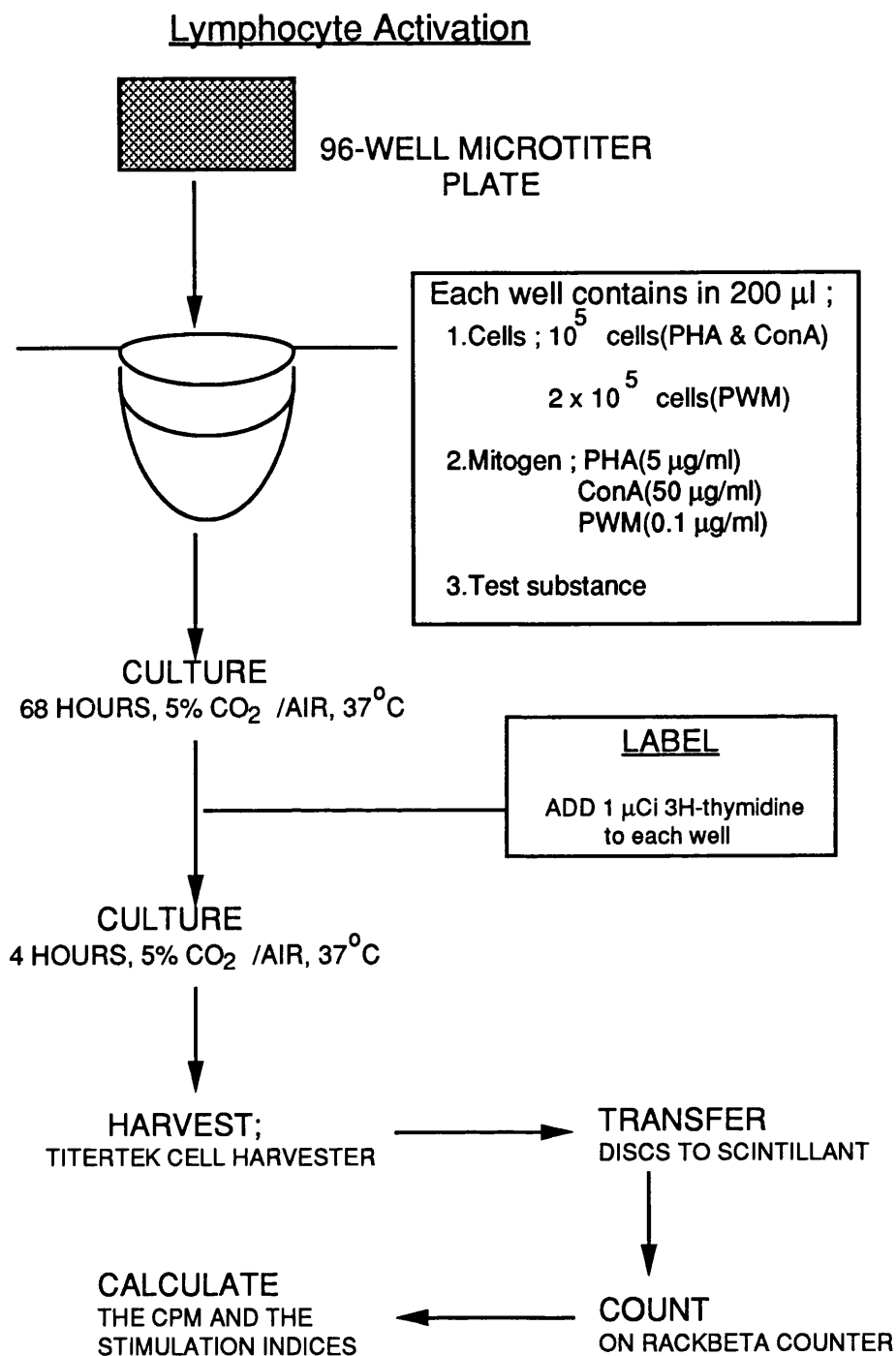


FIGURE 2.7.1.

The protocol for performing the lymphocyte activation assay. The assay has a total culture time of 72 hours. The method for detecting lymphocyte proliferation was thymidine incorporation.

The discs containing the radioactive material were removed from the filter mats and placed in clearly labelled minivials (Pony Vials, Canberra Packard International S.A., Switzerland) and allowed to dry. The scintillation fluid was prepared by adding 4 grams of 2,5 Diphenyloxazole (Scintillation Grade (PPO), United Technologies Packard, U.K.) to one litre of methylbenzene (Pronalys A.R. (toluene), May & Baker, U.K.). Three mls of scintillation fluid were added to each vial, the vials were then counted on a LKB 1216 Rackbeta II liquid scintillation counter (Pharmacia U.K.). The counter was programmed to measure low energy beta emissions.

2.8. THE MEASUREMENT OF INTERLEUKIN - 2 (IL-2).

Assays for IL-2 fall into two main categories : the bioassay and the enzyme linked immunoabsorbant assay (ELISA). For practical as well as historical reasons the bioassay is the assay of choice with most researchers. In general, bioassays measure the proliferative effect of IL-2 on particular responsive cells. In the search for a suitable assay the following responsive cells were evaluated:

- (i) Human lymphoblasts derived from PBMCs stimulated with ConA and cultured for ten days
- (ii) Mouse splenocytes stimulated with ConA and cultured for ten days
- (iii) The murine T-helper cell line, HT-2

Although all three options produced a suitable IL-2 assay (see section 4.3 for evaluation) the HT-2 cell line was the preferred option.

Under normal circumstances ELISAs would be the method of choice for the measurement of IL-2. However, the sensitivity of the commercially available ELISA kits were not as yet comparable to a good bioassay. An in-house ELISA for IL-2 was developed and compared to the commercially available IL-2 ELISA kit (Quantine, R & D Systems, Inc., U.S.A.). The in-house ELISA was no better than the commercially available kit which in turn was not as sensitive as the HT-2 bioassay.

2.8.1. Preparation of IL-2 Supernatants.

Venous blood was collected aseptically into a sterile universal containing enough preservative-free heparin to give a final concentration of 20 units/ml (see section 2.1.). The heparinised blood was mixed with MEM. PBMCs were separated by density gradient centrifugation over Ficoll-Hypaque, washed three times and resuspended in 1 ml complete RPMI 1640 (with HEPES) (see section 2.2.1). The cells were counted and resuspended at 2×10^6 cells/ml (see section 4.4.) in complete RPMI 1640 (with HEPES) supplemented with 4 % FCS. The cells were stimulated with purified phytohaemagglutinin ((PHA), Wellcome Diagnostics, U.K.). The PHA solution was made up in complete RPMI 1640 at 2 μ g/ml (see section 4.4.). The PHA solution and the cells were mixed in a ratio of 1:1 in a 24 well tissue culture cluster (Bibby Sterlin Ltd., U.K.). The cells were cultured for 48 hours at 37 °C in a humidified 5 % CO₂/Air atmosphere. The conditioned media containing IL-2 were harvested by centrifugation at 400 x g for 10 minutes at 4 °C. The supernatants were removed and passed through a 0.22 μ m sterile filter (Millipore U.K.). The IL-2 containing supernatants were stored at -20 °C until assayed.

2.8.2. IL-2 Bioassay.

The responsive cells in the bioassay were the HT-2 cell line which were derived from the murine T helper cells. The cells were maintained in culture by continually seeding at 4×10^4 cells/ml (see section 2.3.1.). Twenty-four hours prior to the assay the cells were split to 10^5 cells/ml in fresh media containing recombinant IL-2. The reasoning behind this was to maximise yield and to ensure that cells going into the assay were in the log phase of their growth curve. On the day of an assay the cells were washed three times in complete RPMI 1640 (with HEPES), resuspended at a cell density of 1.5×10^5 cells/ml in complete RPMI 1640 supplemented with 20 % FCS. The bioassay was set up in 96 well, U-shaped microtiter plates (Bibby Sterlin Ltd., U.K.) (see figure 2.8.1). Eight serial dilutions of the IL-2 containing supernatants (neat, 1:1, 1:3, 1:7, 1:15, 1:31, 1:63, and 1:127) were set up in triplicate down the plate (100 μ l/well). The serial dilutions were carried out in complete RPMI 1640 (with HEPES). Cells (100 μ l) were added to each serial dilution which meant that each serial dilution was diluted by a further 1:1 and that the final FCS concentration was 10 %. The standard curve was constructed by serially diluting recombinant IL-2 ((200 units/ml), B.C.L.) such that eleven dilutions were obtained (200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39 and 0.19 units/ml). Cells were added (100 μ l) to each serial dilution. The bioassay was incubated at 37 °C in a humidified atmosphere of 5 % CO₂/Air for 20 hours. At 20 hours the assay was labelled with ³H-thymidine (see section 2.7.4.) and incubated for a further 4 hours at 37 °C in a humidified atmosphere of 5 % CO₂/Air. The cells were harvested on a Skatron cell harvester (see section 2.7.4.) and the discs were counted on a LKB rackbeta II counter.

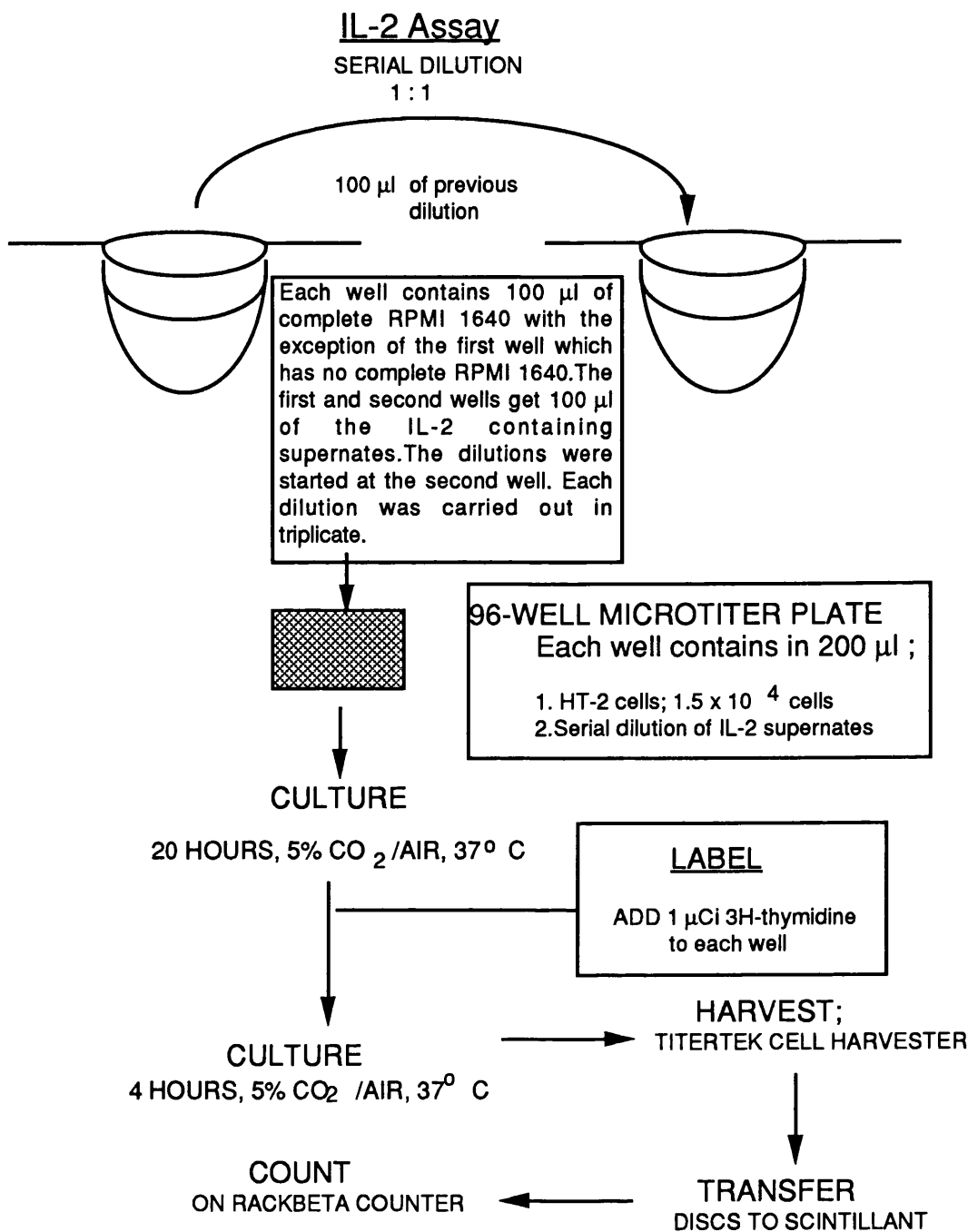


FIGURE 2.8.1.

The protocol for the IL-2 bioassay utilising the HT-2 cell line. The assay has a total culture time of 24 hours. The method for detecting cell proliferation was thymidine incorporation.

2.8.3. Calculation of Results.

A consequence of using the bioassay to measure IL-2 activity in a sample was that a simple extrapolation from a standard curve of one value was not feasible. The solution to the problem of variation due to biological aspects of the assay to some extent was overcome by the use of serial dilutions of the sample and producing an activity curve for each sample. The accepted methodology was that 1 unit of IL-2 activity was the amount required to produce 50 % maximum activity. The sample can then be compared to the standard.

2.9. MEASUREMENT OF INTERLEUKIN - 1 (IL-1).

The wide range of biological activities attributed to interleukin-1 (IL-1) both *in vivo* and *in vitro* are considered essential to mount an immune response. This range of activities have in turn led to a great variety of methods employed to measure the monokine. Probably the most significant of these activities, and for this reason the vast majority of bioassays are based on this activity, was the capacity of IL-1 to stimulate T-cells to produce IL-2. IL-1 also causes an increase in body temperature *in vivo* which may occur to aid the body's immune response to bacteria and viruses by enhancing the effects of IL-1 on T-cell proliferation. Indeed, there have been assays developed that assess IL-1 through increases in body temperature, although these assays are fraught with reproducibility problems.

Like IL-2 assays, the alternative to bioassays for IL-1 are the ELISA's. IL-1 ELISA's are now reaching the stage where they can be considered viable alternatives to the bioassay. The commercially available ELISA's are moving towards the sensitivity and reproducibility of the better IL-1 bioassays.

A highly sensitive two cell bioassay for detection of IL-1 was used. A commercially available ELISA kit (Cistron Biotechnology, Pine Brook, NJ 07058, U.S.A.) for measuring IL-1 β was also employed on some of the samples to double check that none of the effects were assay derived.

2.9.1. Standard IL-1 Preparation.

The cost of recombinant IL-1 was prohibitive in its use as a standard. The solution was to use an in-house IL-1 standard and calibrate this with reference IL-1 standard obtained from the National Institute for Biological Standards and Controls, Blanch Lane, South Mimms, Potters bar, Herefordshire, EN 63 QG. Sufficient in-house standard was prepared to last a significant length of time. PBMC's were obtained from normal venous blood as described previously (section 2.2.1.) with a few modifications. After the initial centrifugation over lymphoprep™, the autologous serum (plasma:MEM(1:1)) was pipetted off into a sterile universal container. The cells were removed and washed three times in MEM. After the final wash the cells were resuspended in RPMI 1640 with HEPES and counted using a haemocytometer. The cell concentration was adjusted to 1.2×10^6 monocytes/ml, this concentration could be obtained in two ways either by assuming a ratio of 1:3 - 4 (monocytes:lymphocytes) and counting the lymphocytes (section 2.2.1.) or by specific staining for the monocytes. The cells were resuspended in RPMI 1640 with HEPES and 10 % autologous serum (5 % final concentration, bearing in mind that the blood was diluted 1:1 with MEM). The monocytes were stimulated with 20 ng/ml LPS and cultured for 18 hours at 37 °C in a humidified atmosphere of 5 % CO₂/Air. At the end of the incubation period the cells were pelleted by centrifugation (400 x g for 10 minutes). The supernatant was carefully removed and sterile filtered through a 0.2 µm single use filter (Millipore (U.K.) Ltd.). The supernatant was assayed as soon as possible, and once standardised, the in-house standard was aliquoted into 0.5 mls. aliquots and stored at -20 °C.

2.9.2. IL-1 Bioassay.

The bioassay of choice was the sensitive two cell bioassay developed by Larrick (Larrick, 1985). The assay system has two distinct advantages over the more commonly used lymphocyte activating factor (LAF) assay, it was highly sensitive and the incubation period was only 24 hours. The principle behind the assay can be described in simple terms. Upon incubation of the LBRM TG6 cell line, a thioguanine resistant mutant of the murine lymphoma cell line LBRM 33, with a suboptimal concentration of PHA and a given concentration of IL-1 the cells produce IL-2. This IL-2 allows the HT-2 cells, an IL-2 dependent cell line derived from murine T-helper cells, to proliferate in response. The LBRM TG6 and the HT-2 cells were cocultured. Normally in bioassays employing two cell lines the first cell line has to be removed before the second cell line is added. However, in this assay both cells can be cocultured thereby lowering the incubation time. This was possible because the 6-thioguanine resistant (hypoxanthine-guanine phosphoribosyl transferase negative) mutant of the LBRM 33 cell line, LBRM TG6, ceases thymidine incorporation in hypoxanthine-azaserine supplemented media. Prior to addition of tritiated thymidine, hypoxanthine and azaserine were added to block DNA synthesis by LBRM TG6 cells. The incorporation of thymidine then reflects only the DNA synthesis by the HT-2 cells thereby a sensitive, short term assay was possible. Both the HT-2 cells and the LBRM TG6 cells were maintained in culture as described in section 2.3.1. and 2.3.2. respectively. It was important to ensure that both cell lines were used in the assay when in the middle of their log growth phase.

The standard IL-1 preparation was diluted one hundred-fold in RPMI 1640 with HEPES. The standard curve was constructed by setting up a further eleven two-fold serial dilutions of the IL-1 standard (1/100). The serial dilutions (100 μ l) were carried out in a round bottomed 96-well microtiter plate (Bibby Sterlin Ltd., U.K.), each dilution being set up in triplicate (see figure 2.9.1). The controls were RPMI 1640 (no suboptimal PHA) and RPMI 1640 (with suboptimal PHA). The cells were counted and the appropriate number of cells pooled together. The pooled cells were pelleted and washed three times in RPMI 1640. The cells were resuspended at 1.5×10^5 HT-2 cells/ml and 1×10^6 LBRM TG6 cells/ml in complete RPMI 1640 supplemented with 20 % F.C.S.. At this point 100 μ l of cells were added to the control wells that contained RPMI 1640 (no suboptimal PHA). PHA was now added to the remainder of the cells at a working concentration of 2 μ g/ml (final concentration in the assay 1 μ g/ml). The cells stimulated with PHA were now added to the remainder of the wells.

The supernatant samples containing IL-1 were diluted five-fold initially. A curve for each sample was constructed by serially diluting ten-fold this initial dilution. Each sample then consisted of four dilutions (100 μ l) each in triplicate (1/5, 1/50, 1/500 and 1/5000). The cells stimulated with the suboptimal PHA were added to each well (100 μ l). The assay was incubated at 37 °C in a humidified atmosphere of 5 % CO₂/Air for 16 hours.

At 16 hours the LBRM TG6 will have had sufficient time to respond to the IL-1. Equal volumes of the azaserine stock (200 μ g/ml, Sigma Chemical Co., U.K.) and hypoxanthine stock (2×10^{-3} M, Sigma Chemical Co., U.K.) were mixed together. Each well receives 20 μ l of this mixture i.e. 10 μ g/ml azaserine and 10^{-4} M hypoxanthine. The assay was incubated for a further four hours at 37 °C in a humidified atmosphere of 5 % CO₂/Air. This concentration of azaserine reduces thymidine incorporation by LBRM TG6 by 99 %.

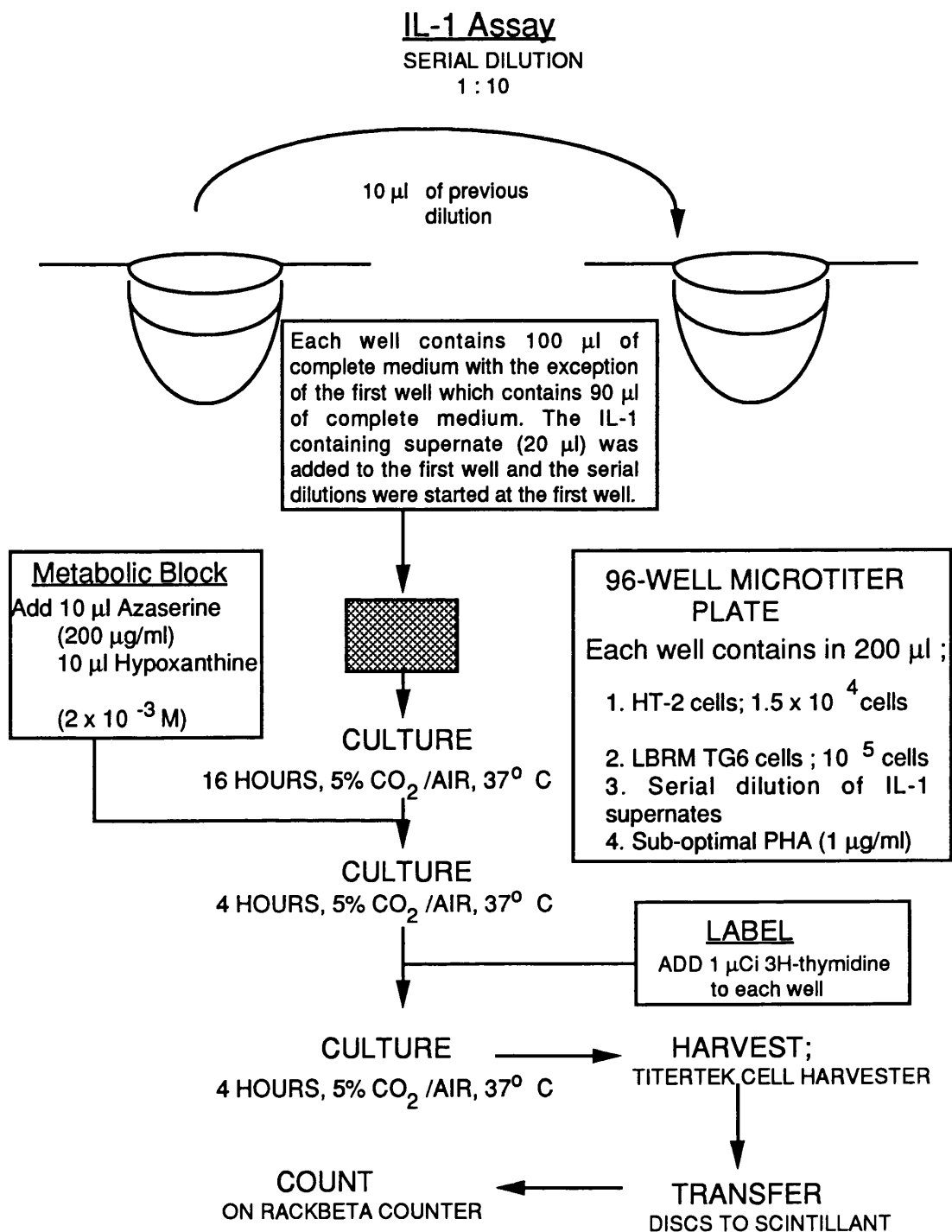


FIGURE 2.9.1.

The protocol for the IL-1 bioassay utilising both the LBRM TG6 and HT-2 cell line (Larrick, 1985). The assay has a total culture time of 24 hours. At 16 hours a metabolic blocking step is used to prevent the LBRM TG6 cell line from proliferating any further. The method for detecting HT-2 cell proliferation was thymidine incorporation.

At 20 hours 1 μCi of tritiated thymidine was added to each well (section 2.7.4.). The tritiated thymidine incorporated was almost exclusively down to the number of HT-2 cells in the S-phase of the cell cycle. The assay was incubated for a further four hours at 37 °C in a humidified atmosphere of 5 % CO_2/Air . The assay was harvested as described previously (section 2.7.4.).

2.9.3. Calculation of IL-1 in samples.

The amount of IL-1 present in a sample was calculated in a similar fashion to IL-2 (section 2.8.3.). A curve was constructed for each sample plotting % maximum against reciprocal of dilution. One unit of activity was defined as the point where 50 % maximum activity was obtained.

2.9.4. IL-1 β ELISA.

The IL-1 β ELISA was supplied in kit form by Cistron Biotechnology, box 2004, 10 Bloomfield Avenue, Pine Brook, NJ 07058, U.S.A.. The kit was supplied as a microtiter plate coated with a monoclonal antibody to IL-1 β (anti-IL-1 β). The ELISA was carried out as outlined in the protocol supplied with the kit. Briefly, the samples and standards were added to the plate and incubated under cover for 2 hours at 37 °C. The plate was washed three times with wash buffer (PBS + 0.02 % Triton X-100 + 0.04 % thimerosal). The second antibody (anti-IL-1 β polyclonal) was now added to the plates and incubated under cover for 2 hours at 37 °C. The plates were washed a further three times with wash buffer. The anti-rabbit IgG - HRP (horse radish peroxidase) conjugate was added to the plates and incubated under cover at room temperature for 30 minutes. The plate was washed three times with the wash buffer. The substrate was added to the plate, the substrate was prepared beforehand by dissolving 2 tablets of o-Phenylenediamine (OPD) in 10 mls deionised water and hydrogen peroxide (70 μ l) was added prior to use. The plate was incubated at room temperature for 15 minutes, after which the reaction was halted by the addition of 50 μ l 4N sulphuric acid. The plate was now read at 490 nm on a Dynatech MR 700 microplate reader.

2.9.5. Calculation of results.

The samples were diluted 1 in 10 in complete RPMI 1640 before being used in the ELISA. The net optical density (OD) was calculated by subtracting the average NSB wells (non-specific binding) from the OD reading obtained for the samples and standards. The standard curve was constructed by plotting the net OD against the IL-1 β concentration. The IL-1 β in each of the samples was then determined by extrapolation from the standard curve.

2.10. THE MEASUREMENT OF INTERLEUKIN - 6 (IL-6).

Interleukin-6 (IL-6) was a comparative newcomer to the cytokine arena. As such the number of bioassays was smaller and the ELISA's were at the developmental stage. IL-6 was originally known as hybridoma growth factor (HGF) and this factor was produced by human mononuclear leukocytes and acts on hybridoma and plasmacytoma cells.

2.10.1. Preparation of IL-6 Supernatants.

The preparation of supernatants containing interleukin-6 (IL-6) was comparatively easy. IL-6 is produced by monocytes and not lymphocytes, there was no need for the monocytes to be stimulated but the cells do require the presence of serum. PBMCs were obtained as previously described (section 2.2.1.). Briefly, the cells were separated by density gradient centrifugation, washed three times with MEM and resuspended in RPMI 1640 with HEPES. The cells were counted and adjusted to a final concentration of 10^6 cells/ml. The PBMCs were cultured in complete media supplemented with 5 % F.C.S. at 37 °C in a humidified atmosphere of 5 % CO₂/Air for 18 hours. The cultures were set up in 24-well clusters (Bibby Sterlin Ltd., U.K.). At the end of the incubation the cells were pelleted (400 x g, 10 minutes, 4 °C) and supernatants sterile filtered. The IL-6 containing supernatants were stored at -20 °C until assayed.

2.10.2. The IL-6 Bioassay.

The bioassay chosen to measure IL-6 was the one based on the B9 cell line. The B9 cells were maintained in culture as described in section 2.3.3.. The standard used in the IL-6 bioassay was recombinant IL-6 (200,000 international units/ml (~ 20 µg/ml), Koch - Light Ltd., Rookwood Way, Haverhill, Suffolk, CB9 8PB). A stock solution of recombinant IL-6 was prepared at 400 units/ml. A standard curve was constructed by serial two-fold dilutions of this stock solution (100 µl in each well (11 dilutions) in triplicate). The samples were also diluted two-fold serially (8 dilutions). The B9 cells were counted, pelleted, washed and resuspended at a working concentration of 10^4 cells/ml. The cells were added to the standard and samples (100 µl). The assay was set up in 96-well round bottomed microtiter plates (Bibby Sterlin Ltd., U.K.) (see figure 2.10.1). The assay was incubated at 37 °C in a humidified atmosphere of 5 % CO₂/Air for 68 hours.

At 68 hours the cells were labelled with tritiated thymidine (section 2.7.4.) and incubated for a further four hours. After 72 hours in culture the assay was harvested on a Skatron cell harvester (section 2.7.4.) and the discs transferred to vials and counted on an LKB Rackbeta II counter.

IL-6 Assay

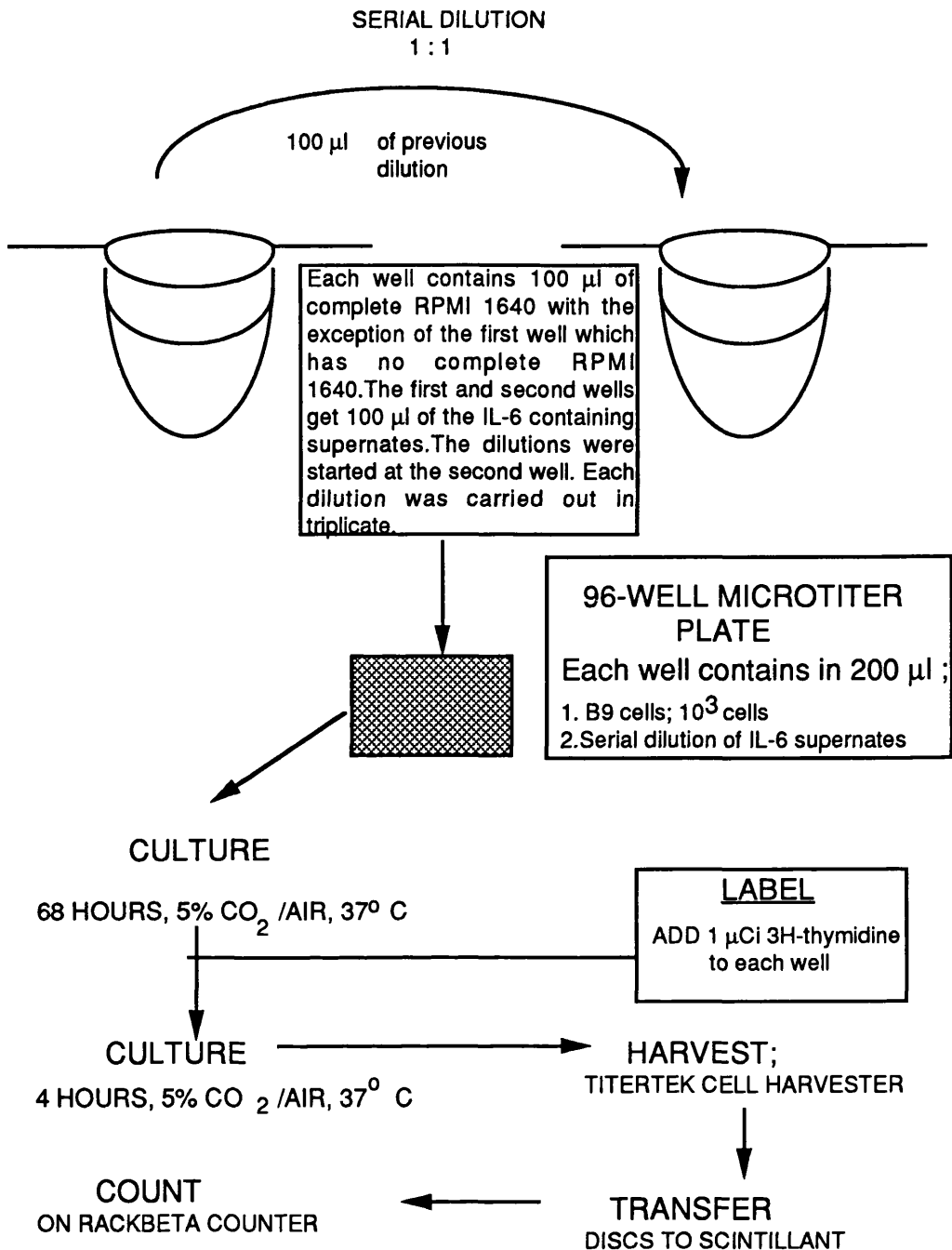


FIGURE 2.10.1.

The protocol for the IL-6 bioassay utilising the B9 cell line (Aarden, 1987). The assay has a total culture time of 72 hours. The method for detecting B9 cell proliferation was either thymidine incorporation or the MTT reaction.

2.10.3. Calculation of IL-6 data.

As in the case of the other bioassays, the samples are serially diluted to produce a curve. The amount of IL-6 was established at the dilution of sample that produced 50 % response. The amount is then adjusted by comparing to the standard curve.

2.10.4. Alternative detection of proliferation.

At 68 hours instead of adding [3H]-thy it was possible in this assay set up to use the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to ascertain the number of live cells. The MTT forms the basis of a colorimetric assay, the system works because active mitochondria possess enzymes (dehydrogenase enzymes) that cleave the tetrazolium ring to yield a coloured product that was purple. MTT was obtained from the Sigma Chemical Co. Ltd. and was dissolved in PBS to give a concentration of 5 mg/ml. The MTT was added to the assay four hours prior to the end of the incubation i.e. at 68 hours. The final concentration of MTT in the assay was 500 µg/ml. The incubation was continued to 72 hours. The reaction was halted by the addition of 50 µl of a stop solution (10 % Triton x-100 and 0.5N HCl). After the purple crystals were dissolved the plate was read on a Dynatech MR 700 microplate reader, using a test wavelength (λ) of 570 nm, a reference wavelength of 630 nm, and a calibration setting of 1.99. The advantage of using this method of detection are two - fold, firstly to cut out the need for radioactivity and secondly the volumes of both cells and samples can be reduced successfully.

2.11. THE MEASUREMENT OF SOLUBLE INTERLEUKIN - 2 RECEPTORS (sIL-2R).

Soluble interleukin-2 receptors (sIL-2R) concentrations are considered to be a good marker for immune system activation. The sIL-2R were thought to be derived from proteolytic cleavage of the membrane receptor for IL-2 (tac subunit, p55). The activation of the immune system leads to the switching on of the expression of the tac subunit which in turn leads to an increase in the amount of sIL-2R released by activated cells into the surrounding fluid whether it be serum or tissue culture fluid.

2.11.1. sIL-2R Enzyme linked Immunoabsorbant assay.

The amount of sIL-2R was measured in sera or in cell free tissue culture supernatants. The assay is carried out using a sIL-2R test kit supplied by T-cell Sciences, Inc., 840 Memorial Drive, Cambridge, MA02139. The Cellfree[®] Interleukin-2 Receptor test kit is based on a sandwich enzyme immunoassay. It was an enzyme linked immunoabsorbant assay (ELISA). Basically, the first anti-IL-2R monoclonal antibody was absorbed onto a polystyrene plate. The sample or standard was added and bound to the coating antibody. An enzyme conjugated anti-IL-2 monoclonal antibody directed against a second epitope on the sIL-2R molecule was then added. The second monoclonal bound to the captured sIL-2R thereby completing the sandwich. The detection involved adding the substrate for the conjugated enzyme and measuring the coloured product that was formed. The amount of colour was in proportion to the amount of sIL-2R present. The reaction was halted at an appropriate time.

2.11.2. sIL-2R; The assay.

The protocol for the Cellfree ® interleukin-2 receptor kit was as provided in the test kit. Briefly, the plate was coated with anti-IL-2R coating antibody, 100 µl of the coating antibody solution was added to each well. The plate was covered and incubated at 4 °C for 18 hours (overnight). The coating antibody solution was discarded. The unoccupied binding sites on the plastic were now saturated with a blocking buffer. The blocking buffer usually contains a protein that binds and fills the unoccupied sites. The blocking buffer in this case was as supplied by the manufacturer. Each well was covered and incubated with 300 µl of blocking buffer for 2 hours at 37 °C. The plates were now ready to use in the assay.

The blocking buffer was discarded and the wells were washed three times with 350 µl washing buffer. The washing buffer was made up as per manufacturers instruction from a 10 x stock, it was a PBS based buffer. The plates were now ready to receive the samples and standards. There were five standards, 0, 100, 400, 800 and 1600 units/ml. The standards were carried out in duplicate. Two wells were set aside as blanks. Each well used in the assay received 100 µl of sample diluent except for the two blank wells.

The sample diluent was supplied by the manufacturer and was serum protein in a buffered solution. The standards (50 µl) were added in duplicate to the appropriate wells, and the samples (50 µl) were added to the remainder of the wells designated to the samples. The plate was agitated to mix the contents of each well thoroughly. The plates were covered and incubated at 37 °C for 2 hours. The standards and samples were discarded and the wells were washed three times with washing buffer (350 µl). The HRP - conjugated antibody (100 µl) was added to all wells with the exception of the blanks and incubated at 37 °C for 2 hours. About 5 minutes before the end of the incubation the substrate solution was made up. Two O-phenylenediamine (OPD) tablets were dissolved in 20 mls of substrate diluent (supplied). The substrate diluent contains hydrogen peroxide in a buffered solution. The plates were removed from their incubation and the contents of the wells discarded, the wells were washed three times with 350 µl washing buffer. All wells including the blank wells receive 100 µl of the OPD substrate solution. The plates were incubated at room temperature for 30 minutes. The reaction was stopped by the addition of 50 µl of the stop solution. The stop solution was 2N sulphuric acid (H_2SO_4) and was prepared by adding 5.8 mls of concentrated H_2SO_4 to 94.2 mls distilled water. The plates were now read at 490 nm against the substrate blank on a Dynatech MR 700 microplate reader. The plate have to be read within two hours of adding the stop solution.

2.11.3. Calculation of Results.

A standard curve was constructed by plotting the concentration of the standard sIL-2R in units/ml against the absorbance at 490 nm. The amount of sIL-2R in each sample was then calculated using the standard curve.

2.12. CHARACTERISATION OF MESSENGER RNA (mRNA).

Ribonucleic acid (RNA) has a central role to play in the proposed mode of action of the steroid hormone and clearly messenger RNA (mRNA) is the site of the primary change in going from a steady state to a regulated state. The mRNA in reality represents the phenotype of a cell and as such is a manifestation of the cellular genotype and the environment in which the cell finds itself. It is therefore important to characterise the mRNA of a controlled cell to determine the importance of regulatory hormones.

2.12.1. Isolation of RNA.

There have been many procedures for the extraction of RNA, but on the whole the methods of choice are usually based on the Guanidinium extraction method pioneered by Cox (Cox, 1968). Recently it has become possible to extract RNA without the use of Caesium Chloride cushions favoured by Chirgwin (Chirgwin, 1979). The method chosen was a single step isolation by Acid Guanidinium Thiocyanate Phenol - Chloroform (AGPC) extraction (Chomczynski, 1987).

Basically a denaturing solution was prepared that contains 4 M guanidinium thiocyanate (Fluka Chemicals Ltd., Peakdale Road, Glossop, Derbyshire SK 13 9 XE.), 25 mM sodium citrate (BDH) pH 7.0, 0.5 % sarcosyl (Sigma Chemical Co. Ltd.) and 0.1 M 2 - β - mercaptoethanol (Sigma Chemical Co. Ltd.). Cells were washed with cold PBS and pelleted. The cell pellet ($\sim 10^7$ cells) was mixed with 1 ml denaturing solution. The cell pellets / denaturing solutions were hand homogenised (about 20 strokes). After the cells were disrupted, 100 μ l of 2 M sodium acetate (BDH) pH 4.0 was added and the solution mixed. At this point 1 ml of water saturated phenol (Rathburn Chemicals Ltd.) was added to the mixture. Finally, 200 μ l of chloroform / isoamyl alcohol (49 : 1) was added and the solution was vortexed for 10 seconds. The solution was incubated on ice for 15 minutes.

The solution was transferred to microcentrifuge tubes and the tubes were centrifuged in a microcentrifuge at full speed for 15 minutes in the cold room (4 °C). The solution separates into two distinct phases, the aqueous phase contains the RNA and the phenolic phase which contains the protein and the DNA. The aqueous phase was pipetted off into fresh microcentrifuge tubes (~ 0.5 mls). To the aqueous phase, 0.5 mls of 'cold' isopropanol was added. The RNA was precipitated out by incubating this solution on dry ice for 30 minutes. The precipitate was pelleted by centrifugation in a microcentrifuge at full speed for 15 minutes in the cold room. The supernatant was discarded and the RNA pellet was taken up in denaturing solution. At this stage the samples were pooled to give a final volume of 300 µl RNA in denaturing solution.

The RNA was then precipitated out again by adding 2 volumes of cold ethanol (600 µl) and incubating on dry ice for 30 minutes. The RNA was pelleted by centrifugation on a microcentrifuge for 10 minutes in the cold room. The RNA pellet was washed twice with 1 ml of 'cold' 70 % ethanol. The pellet was then lyophilised and resuspended in 60 µl of sterile water and stored at - 20 °C.

2.12.2. Assessing Quantity and Quality of RNA.

The RNA preparation was run on a 1 % agarose (Gibco BRL) gel to check the quality of the RNA. This allows visualisation of in particular the two distinct bands at 18S and 28S which are produced by the ribosomal RNA (constitutes 80 - 85 % of total RNA). There is also a band at 5S but this band is more difficult to see. The amount of RNA can be quantified in two distinct ways. The first method is to use the DNA dipstick™ kit (distributed by British Biotechnology Ltd., Watlington Road, Cowley, Oxford OX 4 5LY.). In this procedure it was a case of spotting some nucleic acid onto a strip and following the manufacturers instructions and reading the colour of the spot after treatment. This method has the advantage that it uses very little sample and the disadvantage that it is an estimation by eye of the concentration of a dilution and relies on picking the right dilution to measure. The second method is the most commonly used, it relies on the optical density (OD) of RNA at 260 nm. The advantage of this method is that from the OD an accurate concentration can be calculated. The major disadvantages are that the volume of sample is relatively high and the sample once measured has usually then to be utilised in the actual analysis of the RNA.

2.12.3. Northern blot analysis of RNA.

The accepted procedure for analysing RNA is the Northern blot. The northern blot was patterned on the Southern blot hence its name. Essentially the nature of the technique is to denature the secondary structure of the RNA by mixing the RNA with an agent such as formaldehyde that breaks the hydrogen bonds between the base pairs. The outcome of this is that the RNA is unfolded and becomes linear. The RNA is then subjected to gel electrophoresis, nitrocellulose blotting, hybridisation with a labelled DNA sequence complementary to the RNA being studied. The northern blot indicates both the size and the presence of a specific mRNA. It also can indicate the amount relative to other mRNAs. The procedure is ideal when comparing the amounts of specific RNA produced in cells under different conditions.

The RNA sample was prepared for gel electrophoresis as outlined in the protocol provided with the Hybridisation transfer membrane (Genescreen Plus™, Dupont). Briefly the required amount of RNA was dissolved in an appropriate solution containing : 50 % formamide (deionised)(Gibco BRL), 6 % formaldehyde (BDH, U.K.) and 12 mM Tris - 6 mM sodium acetate - 0.3 mM EDTA, pH 7.5 (TAE buffer). This solution was incubated at 60 °C for 15 minutes. The RNA solution was transferred to ice and stored until loaded onto the gel. The gel was prepared by adding 1.5 g of agarose (Gibco BRL, U.K.) to 110 mls of distilled water and microwaved until dissolved. The gel solution was allowed to cool at room temperature until the temperature of the gel solution reached 60 - 65 °C. At this point 15 mls of 120 mM Tris - 60 mM sodium acetate - 3 mM disodium ethylenediaminetetra - acetate (sodium EDTA), pH 7.5 (10 x TAE) and 24.3 mls of formaldehyde (final concentration 6 %, BDH, U.K.) were added to the gel solution ; the solution was mixed and poured. The gel solution was poured onto an appropriate gel cast and allowed to set. The gel was placed in a gel tank that contained the electrophoresis buffer ; 12 mM Tris - 6 mM sodium acetate - 0.3 mM sodium EDTA, pH 7.5 (TAE). The tracking dye was added (5 µl) to each of the RNA samples (0.01 %

Orange G in 50 % glycerol and 50 % 2 x TAE buffer). The electrophoresis was set up to run for 210 minutes at a voltage of 80 V.

The gel was removed from the gel tank and rinsed briefly with distilled water to remove excess formaldehyde. The gel was incubated in 50 mM sodium hydroxide for 30 minutes to aid the transfer of larger RNA. The gel was neutralised by incubating the gel in 50 mM tris, pH 7.0 for 30 minutes. The gel was now ready to blot. The genescreen plus™ was cut to size, the bottom right hand corner was marked (care was taken to mark the correct side). The genescreen plus™ was wet with distilled water. The membrane was then soaked in 1.5 M sodium chloride - 0.15 M sodium citrate (10 x SSC) for 15 minutes. A large glass plate was placed on the bench and 2 pieces of filter paper (Whatman 3MM) were cut to the same size of this plate. The plate was wet with 10 x SSC and the filter papers were placed on the glass plate soaking up the 10 x SSC and acting as a reservoir. The gel was now placed on top of the filter paper making sure that there were no air bubbles and spacers were placed all round the gel. The membrane was placed on top of the gel again making sure that there were no air bubbles. Two pieces of filter paper identical in size to the gel were then placed on top of the membrane. A stack of tissues were placed on top of the filter paper and a small weight was placed on top. The transfer was allowed to proceed overnight (about 18 hours). The membrane was removed carefully and allowed to dry at room temperature. The formaldehyde reaction was reversed by baking at 80 °C for two hours.

The blot was probed with commercially available probes that were labelled in the laboratory with radioactive phosphorus (P^{32}). The IL-2 probe was a probe cocktail, an equimolar mixture of four exons each 30 or 31 bases in length, supplied by British Biotechnology Ltd., U.K.. The IL-2R (Pr - 1) probe was a 40 base antisense synthetic oligonucleotide supplied by Oncogene Science, Inc., U.S.A.. The abl probe was provided by Dr J. W. Gow, Department of Neurology, Southern General Hospital, Glasgow. The probes were labelled by an 5' end labelling technique. The probe to be labelled was incubated with the enzyme T4 Polynucleotide kinase (1 μ l), T4 kinase buffer (21 μ l) (including excess Adenosine diphosphate (ADP)) and P^{32} labelled

Adenosine triphosphate (3 μ l) (ATP). The reaction mixture was incubated for 45 minutes at 37 °C. At the end of the reaction 170 μ l of sterile water was added to the mixture. The labelled ATP that was not exchanged was separated from the labelled RNA by column chromatography. The reaction mixture was passed down a G 50 sephadex column (Pharmacia LKB Biotechnology) and the labelled probe fraction collected. The unexchanged ATP was retained in the column whereas the probe passed through relatively quickly. The column was washed with a further 150 μ l sterile water to flush out as much of the hot probe as possible.

The nitrocellulose blot was sealed into a plastic bag together with 5 mls of the hybridisation solution (SSPE (20 x : 175.3g (3 M) sodium chloride, 27.6g (0.18 M) sodium dihydrogen phosphate and 7.4g (0.02 M) sodium EDTA pH 7.4), SDS (10 % sodium dodecyl sulphate) and salmon sperm DNA) and incubated for 15 minutes at 63 °C. This is known as the prehybridisation period and the object of this is to block any unoccupied sites with salmon sperm DNA. The labelled probe was then added to the bag containing the blot and the bag was resealed and incubated at 63 °C overnight.

The blot was removed from the bag transferred to a sandwich box and washed with 5 x SSPE : 0.1 % SDS for 20 minutes at room temperature. The blot was then washed in 1 x SSPE : 0.1 % SDS for 20 minutes at room temperature. The blot was removed from the box and excess liquid was shaken off and monitored to check the background. The blot was placed in bag and sealed in order to retain the moisture. The blot was autoradiographed i.e. placed in a cassette together with film at - 70 °C. The film was developed and the blot studied.

CHAPTER 3.

LYMPHOCYTE ACTIVATION - THE EFFECT OF CALCIOTROPHIC AND SEX HORMONES.

3.1. LYMPHOCYTE ACTIVATION.

The stimulation of peripheral blood mononuclear cells (PBMC), T and B cells, by non specific mitogens or specific antigens provides an insight into immune function. Lymphocyte activation is usually seen as the initial stage in evaluating the effects of certain parameters on the immune system. The lymphocyte activation assay has the advantage of being rapid, reproducible and the assay requires a relatively small amount of peripheral blood.

The method used to detect lymphocyte activation was incorporation of tritiated thymidine into DNA, which reflects accurately the number of lymphocytes in the S-phase of the cell cycle. Cell proliferation may be required for lymphocyte differentiation into effector cells, however, care was necessary when interpreting these results as cell division is not considered a function per se. Lymphocyte activation can generate information about cell phenotype and function.

3.1.1. Mitogen induced Lymphocyte activation.

Over the last thirty years mitogens have become the agents of choice when it comes to assessing lymphocyte activation. Nowell (Nowell, 1960) laid the groundwork for this when he discovered that phytohaemagglutinin (PHA), a lectin extracted from kidney bean, could transform small lymphocytes into proliferating lymphoblasts in cell culture. Since then a number of other mitogens have been identified that can stimulate a sizeable proportion of the lymphocyte population. These lectins accomplish lymphocyte activation by binding to specific sugar moieties on the lymphocyte membrane, and therefore activation can be inhibited by the addition of the free sugar. Mitogens can be characterised into T-cell mitogens, B-cell mitogens or mitogens that act on both T and B cell populations. The largest proportion of cells within peripheral blood are the T-lymphocytes and as a result T-cell mitogens are the best characterised and utilised. The most routinely used mitogens to evaluate lymphocyte activation were PHA, concanavalin A (Con A) and pokeweed mitogen (PWM).

3.2. MITOGENS.

Mitogens are defined as substances that have the ability to induce blast-cell transformation and mitosis in a manner similar to antigens. The mitogen binds to a specific cell surface receptor and the signal thus generated causes a chain of events in the nucleus culminating in the lymphocyte entering the growth phase of the cell cycle. A large proportion of the mitogens that have been characterised are derived from plant proteins. These substances are more commonly referred to as lectins. These lectins have binding sites with a specificity for particular sugars. Whereas antigens stimulate a specific colony of cells, mitogens stimulate a large proportion of lymphocytes.

3.2.1. Phytohaemagglutinin (PHA).

Phytohaemagglutinin has been the most extensively studied mitogen. As mentioned previously it is a lectin extracted from kidney beans (*phaseolus vulgaris*). The PHA preparation used for lymphocyte activation has a molecular weight of 126 Kd. The protein consists of four subunits. The lectin recognises oligosaccharide on the surface of the lymphocyte.

3.2.2. Concanavalin A (Con A).

Concanavalin A is a lectin extracted from Jack beans (*canavalia enisformis*), it constitutes 3-4 % of Jack bean proteins. Con A has a molecular weight of 102 Kd and is a tetramer. It binds residues of α -D-mannopyranose and α -D-glucopyranose with unmodified hydroxyl groups at C3, C4 and C6. Con A also has binding sites for Ca^{++} and for a transition metal ion such as Mn^{++} . Con A will not bind to the sugar residues until the two metal binding sites (in each subunit) are filled. The binding of these ions facilitates a conformational change that allows the binding of the specific sugars.

3.2.3. Pokeweed Mitogen (PWM).

Pokeweed mitogen was extracted from pokeweed roots (*Phytolacca americana*), it has a molecular weight of 32Kd. PWM was a monomer and has an affinity for N-acetyl- β -glucosamine oligomers.

3.2.4. Lymphocytes - The target for mitogens.

Both PHA and Con A are termed T-cell mitogens, and are polyclonal in nature as they react with the lymphocyte cell surface non-specifically (i.e. not as an antigen). They perform the same task as an antigen but target a larger population. As a consequence of the sugar specificity of these mitogens it appears that T-helper cells are preferentially stimulated by PHA and T-suppressor cells by Con A. However, the differences are not as clear cut as PHA has a specificity for oligosaccharides. PWM on the other hand activates B-cells but this occurs only in the presence of T-cells and is therefore considered a T-cell dependent B-cell mitogen.

3.3. STANDARDISATION OF MITOGEN CONCENTRATION FOR OPTIMAL LYMPHOCYTE ACTIVATION.

3.3.1. The standardisation of the lymphocyte activation assay.

A lymphocyte activation assay was set up (section 2.7.) with peripheral blood mononuclear cells from 7 normal volunteers. The PBMCs were cultured in the presence of a range of concentrations for each of the mitogens. The PHA was set up at the final concentrations of 10, 5, 2 and 1 µg/ml, likewise the Con A was set up at 100, 50, 20 and 10 µg/ml. PHA and Con A were added to cultures that contained cells at 5 x 10⁵ cells/ml. PWM was added to cells that had a cell density of 10⁶ cells/ml.

The results were expressed both as counts per minute (cpm) and as stimulation indices (SI) according to the formulae;

$$\text{stimulation index} = \frac{\text{mean cpm mitogen} - \text{mean cpm control}}{\text{mean cpm control}}$$

3.3.2. The optimal concentrations of PHA, Con A and PWM.

Table 3.3.1. records the variation in the cpms and stimulation indices at the various concentrations of the three mitogens. Each result was expressed as the mean of 7 normal individuals with the standard error of the mean (SEM) in parenthesis.

Standardisation of mitogen concentration for
optimal lymphocyte activation

PHA μg/ml	CPM (x 10 ⁻²)	SI (x 10 ⁻²)	ConA μg/ml	CPM (x 10 ⁻²)	SI (x 10 ⁻²)	PWM μg/ml	CPM (x 10 ⁻²)	SI (x 10 ⁻²)
10	73.8 (10.3)	0.58 (0.07)	100	28.3 (2.7)	0.22 (0.02)	0.5	30.5 (3.3)	0.23 (0.03)
5	77.8 (10.2)	0.61 (0.07)	50	55.5 (10.9)	0.43 (0.07)	0.1	33.2 (3.9)	0.25 (0.03)
2	38.2 (5.4)	0.30 (0.02)	20	29.6 (4.8)	0.23 (0.03)	0.05	15.3 (1.2)	0.11 (0.02)
1	12.5 (2.1)	0.09 (0.02)	10	21.3 (3.8)	0.16 (0.02)	0.01	7.9 (1.1)	0.05 (0.01)

TABLE 3.3.1.

The table presents the data from a set of experiments designed to optimise the concentrations of mitogens required for lymphocyte activation. The data were presented as both the cpm and also the stimulation index. The data were the mean of 7 normal volunteers with the standard error of the mean (SEM) in parenthesis.

The data clearly demonstrates that the optimum concentration for the stimulation of PBMCs by the three mitogens were as follows;

PHA 5 µg/ml

Con A 50 µg/ml

PWM 0.1 µg/ml

These concentrations of mitogen were used in the subsequent assays for lymphocyte activation.

3.4. CALCIOTROPHIC HORMONES - A ROLE IN THE IMMUNE SYSTEM ?

The demonstration that 1,25 dihydroxyvitamin D₃ receptors are present on activated T and B cells suggested a wider role for the metabolite (Provvedini, 1983; Bhalla, 1983). A possible role in the immune system was postulated by Tsoukas et al, when 1,25(OH)₂D₃ was shown to inhibit interleukin-2 activity (Tsoukas, 1984). 1,25(OH)₂D₃ was not the only calciotropic hormone with receptors in cells of the immune system. In 1974, Marx et al (Marx, 1974) demonstrated that cultured human lymphocytes expressed calcitonin receptors. This observation was followed some years later by the discovery of parathyroid hormone receptors on circulating bovine lymphocytes (Yamamoto, 1983; Perry III, 1984). An explanation for these observations has not been forthcoming. In view of these findings a study was set up to look at the effects of five calciotropic hormones on lymphocyte activation. The five calciotropic hormones studied were;

1,25 dihydroxyvitamin D ₃	(1,25(OH) ₂ D ₃)
24,25 dihydroxyvitamin D ₃	(24,25(OH) ₂ D ₃)
25 hydroxyvitamin D ₃	(25(OH)D ₃)
bovine (1-35) parathyroid hormone	(b(1-35)PTH)
salmon calcitonin	(sCT)

3.4.1. Lymphocyte activation in the presence of calciotropic hormones.

PBMCs were obtained by density gradient centrifugation (section 2.2.1.) from ten normal healthy individuals. The cells were set up for a lymphocyte activation assay (section 2.7.) in the presence of a range of calciotropic hormones (section 2.5.). Each well in the 96 well microtiter plate contained 10^5 PBMCs (2×10^5 PBMCs in the case of PWM), PHA ($5 \mu\text{g/ml}$) or Con A ($50 \mu\text{g/ml}$) or PWM ($0.1 \mu\text{g/ml}$), and a given concentration of one of the calciotropic hormones. Each test was carried out in triplicate. The tests are incubated at 37°C in a humidified atmosphere of 5 % CO_2/Air for 68 hours. At 68 hours, $1 \mu\text{Ci}$ of tritiated thymidine was added to each well and the tests were incubated for a further four hours. The results were expressed as counts per minute of tritiated thymidine incorporated and as stimulation indices.

3.4.2. 1,25(OH)₂D₃ - The effective inhibitor.

The effects of the five calcitrophic hormones (1,25(OH)₂D₃, 24,25(OH)₂D₃, 25(OH)D₃, b(1-35)PTH and sCT) on lymphocyte activation were documented in tables 3.4.1., 3.4.2., 3.4.3., 3.4.4. and 3.4.5. respectively. The first impression from the tabulated data was, that of the calcitrophic hormones studied, only 1,25(OH)₂D₃ had a significant inhibitory effect on lymphocyte activation. PTH and CT appeared to enhance lymphocyte activation but this effect never reached significance, however, 24,25(OH)₂D₃ and 25(OH)D₃ did appear eventually to inhibit lymphocyte activation. 24,25(OH)₂D₃ and 25(OH)D₃ appeared to inhibit at higher concentrations (10⁻⁷ M) but this also did not achieve statistical significance.

Closer inspection of the cpm presented in table 3.4.1. reveals that 1,25(OH)₂D₃ inhibits lymphocyte activation stimulated by all three mitogens. Whereas the inhibition of PHA - induced lymphocyte activation by 1,25(OH)₂D₃ achieves significance at 10⁻¹⁰ M, the inhibition of Con A - and PWM - induced lymphocyte activation does not achieve significance until 10⁻⁹ M (PWM) and 10⁻⁸ M (Con A). The stimulation data similarly achieves statistical significance confirming this observation. The data for 1,25(OH)₂D₃ is presented as a graph in figure 3.4.1.. The normal physiological range for 1,25(OH)₂D₃ concentration in serum occurs between 10⁻¹¹ M and 10⁻¹⁰ M ; only the inhibition of PHA induced activation occurs within this range.

Tables 3.4.2. and 3.4.3. present the effects of 24,25(OH)₂D₃ and 25(OH)D₃ on lymphocyte activation. In each case there was no significant difference in activation whether the cells were stimulated with PHA, Con A or PWM. However, there appeared to be a trend that suggested a slight inhibition of activation at higher concentrations of these metabolites. The normal serum concentrations for these metabolites are between 10⁻¹⁰ M to 10⁻⁹ M for 24,25(OH)₂D₃ and between 10⁻⁸ M and 10⁻⁷ M for 25(OH)D₃.

Lymphocyte Activation induced by PHA, Con A and
PWM in the presence of 1,25 dihydroxyvitamin D₃

1,25(OH) ₂ D ₃	PHA 5µg/ml		ConA 50µg/ml		PWM 0.1µg/ml	
(Molar)	CPM (x 10 ⁻³)	SI (x 10 ⁻³)	CPM (x 10 ⁻³)	SI (x 10 ⁻³)	CPM (x 10 ⁻³)	SI (x 10 ⁻³)
0	28.4 (2.2)	0.84 (0.14)	17.7 (1.6)	0.54 (0.11)	14.7 (1.9)	0.24 (0.06)
10 ⁻¹³	28.9 (2.1)	1.14 (0.28)	19.0 (1.5)	0.78 (0.20)	15.1 (2.0)	0.33 (0.09)
10 ⁻¹²	28.8 (2.0)	1.02 (0.22)	18.3 (1.4)	0.65 (0.15)	15.1 (2.1)	0.30 (0.08)
10 ⁻¹¹	27.4 (1.9)	0.95 (0.20)	18.5 (1.3)	0.67 (0.15)	14.7 (1.9)	0.29 (0.08)
10 ⁻¹⁰	22.9* (1.8)	0.83* (0.19)	18.1 (1.4)	0.64 (0.15)	13.7 (1.7)	0.26 (0.06)
10 ⁻⁹	14.7* (1.1)	0.58* (0.11)	14.7 (1.0)	0.59 (0.12)	12.8* (1.4)	0.27* (0.06)
10 ⁻⁸	12.7* (1.0)	0.51* (0.08)	13.3* (1.0)	0.55* (0.10)	12.7* (1.4)	0.31* (0.07)
10 ⁻⁷	14.9* (1.2)	0.61* (0.13)	13.8* (0.9)	0.58* (0.12)	13.7* (1.5)	0.32* (0.09)

TABLE 3.4.1.

The table presents the data from an experiment designed to show the effect of 1,25(OH)₂D₃ on either PHA, Con A or PWM induced lymphocyte activation. The data were presented as both the cpm of tritiated thymidine incorporated and the stimulation index (SI) for each 1,25(OH)₂D₃ concentration. The data were the mean of 10 normal volunteers with the standard error of the mean (SEM) in parenthesis. The statistical analysis carried out on the data was a paired Wilcoxon test (* p < 0.05) comparing 0 with all other concentrations of 1,25(OH)₂D₃.

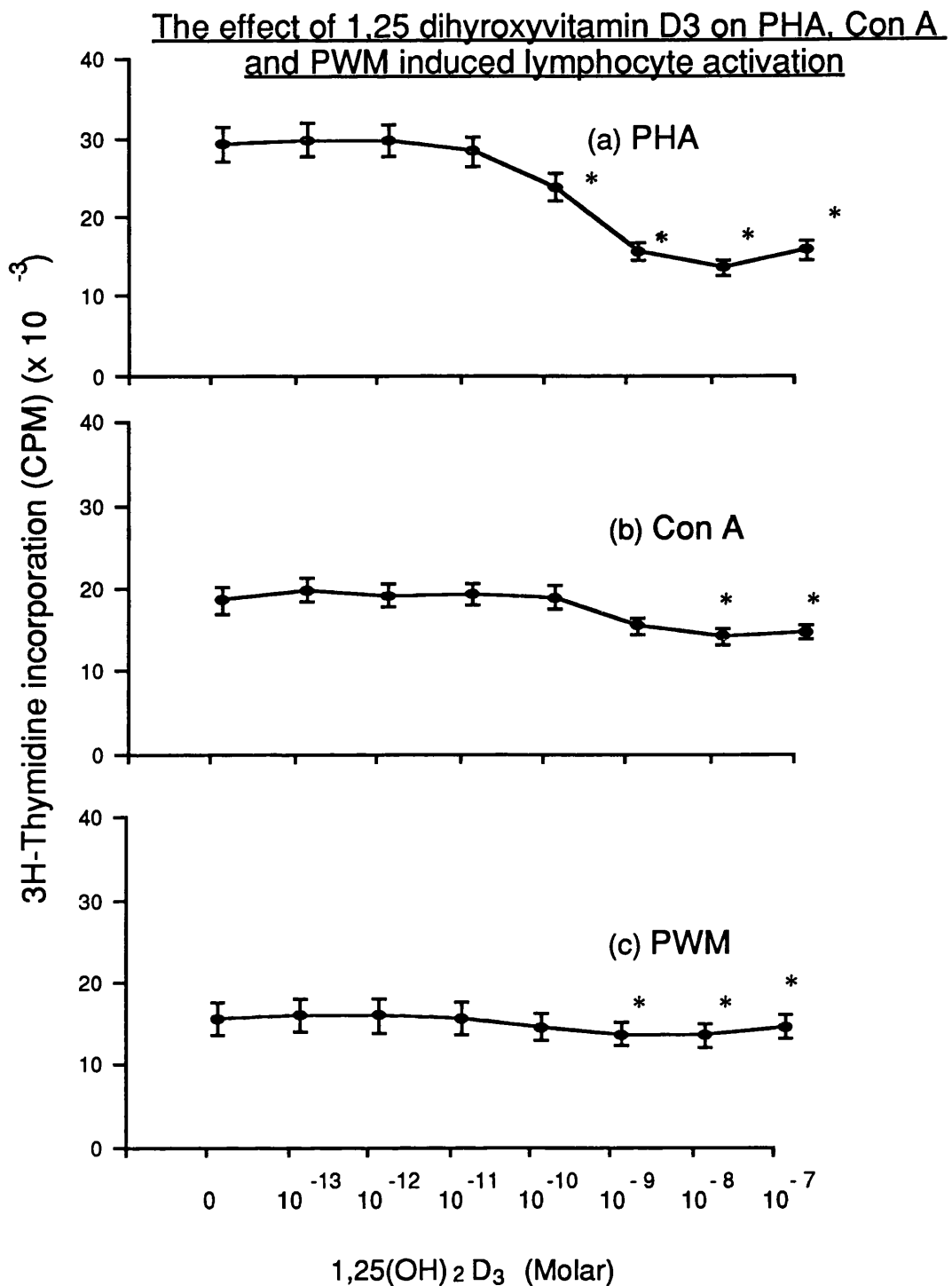


FIGURE 3.4.1.

The figure represents the data from table 3.4.1. in the form of a graph. The data points in each figure are the mean of 10 normal individuals and the error bars represent the standard error of the mean (SEM). The data are cpm of tritiated thymidine incorporated. The data was subjected to a paired Wilcoxon statistical test (* $p < 0.05$) comparing 0 to all other concentrations of 1,25 dihydroxyvitamin D₃.

Lymphocyte Activation induced by PHA, Con A and
PWM in the presence of 24,25 dihydroxyvitamin D₃

24,25(OH) ₂ D ₃	PHA		ConA		PWM	
	5µg/ml		50µg/ml		0.1µg/ml	
(Molar)	CPM (x 10 ⁻³)	SI (x 10 ⁻³)	CPM (x 10 ⁻³)	SI (x 10 ⁻³)	CPM (x 10 ⁻³)	SI (x 10 ⁻³)
0	31.6 (2.0)	0.91 (0.13)	19.7 (1.7)	0.54 (0.05)	15.4 (2.3)	0.22 (0.03)
10 ⁻¹³	30.0 (2.0)	0.96 (0.18)	19.3 (1.2)	0.63 (0.14)	15.2 (2.1)	0.28 (0.09)
10 ⁻¹²	28.3 (2.2)	0.95 (0.18)	18.2 (1.2)	0.60 (0.13)	13.7 (1.3)	0.23 (0.05)
10 ⁻¹¹	26.5 (1.8)	0.95 (0.15)	17.7 (1.4)	0.66 (0.14)	13.7 (1.8)	0.27 (0.07)
10 ⁻¹⁰	27.7 (2.2)	1.00 (0.21)	18.0 (1.4)	0.65 (0.15)	14.3 (1.9)	0.29 (0.08)
10 ⁻⁹	28.5 (1.9)	0.92 (0.20)	19.1 (1.3)	0.59 (0.13)	14.8 (1.7)	0.25 (0.06)
10 ⁻⁸	30.2 (2.2)	0.97 (0.22)	19.5 (1.4)	0.65 (0.17)	15.7 (1.9)	0.29 (0.09)
10 ⁻⁷	34.1 (2.5)	0.92 (0.23)	22.3 (1.5)	0.55 (0.14)	16.3 (2.1)	0.20 (0.04)

TABLE 3.4.2.

The table presents the data from an experiment designed to show the effect of 24,25(OH)₂D₃ on either PHA, Con A or PWM induced lymphocyte activation. The data were presented as both the cpm of tritiated thymidine incorporated and the stimulation index (SI) for each 24,25(OH)₂D₃ concentration. The data were the mean of 10 normal volunteers with the standard error of the mean (SEM) in parenthesis. The data were subjected to a paired Wilcoxon test (* p < 0.05) comparing 0 with all other concentrations of 24,25(OH)₂D₃.

Lymphocyte Activation induced by PHA, Con A and
PWM in the presence of 25 Hydroxyvitamin D₃

25(OH) ₂ D ₃	PHA 5µg/ml		ConA 50µg/ml		PWM 0.1µg/ml	
(Molar)	CPM (x 10 ⁻³)	SI (x 10 ⁻³)	CPM (x 10 ⁻³)	SI (x 10 ⁻³)	CPM (x 10 ⁻³)	SI (x 10 ⁻³)
0	33.9 (3.1)	1.0 (0.22)	20.6 (2.0)	0.63 (0.15)	16.5 (2.2)	0.26 (0.07)
10 ⁻¹³	28.4 (2.3)	0.80 (0.13)	19.7 (1.3)	0.53 (0.07)	16.1 (2.1)	0.21 (0.02)
10 ⁻¹²	28.3 (2.2)	0.86 (0.20)	18.3 (1.5)	0.58 (0.15)	15.5 (2.0)	0.24 (0.04)
10 ⁻¹¹	27.2 (2.4)	0.86 (0.13)	18.5 (1.6)	0.61 (0.12)	15.1 (2.0)	0.25 (0.06)
10 ⁻¹⁰	28.2 (2.1)	1.05 (0.17)	18.8 (1.5)	0.74 (0.14)	15.3 (2.0)	0.32 (0.06)
10 ⁻⁹	28.9 (2.3)	0.90 (0.13)	20.1 (1.5)	0.69 (0.14)	16.0 (2.0)	0.29 (0.06)
10 ⁻⁸	31.2 (2.4)	0.86 (0.20)	21.0 (1.8)	0.64 (0.18)	16.8 (2.1)	0.26 (0.08)
10 ⁻⁷	34.4 (2.6)	1.16 (0.18)	23.4 (2.3)	0.82 (0.17)	16.0 (1.6)	0.31 (0.06)

TABLE 3.4.3.

The table presents the data from an experiment designed to show the effect of 25(OH)₂D₃ on either PHA, Con A or PWM induced lymphocyte activation. The data were presented as both the cpm of tritiated thymidine incorporated and the stimulation index (SI) at the various concentrations of 25(OH)₂D₃. The data were the mean of 10 normal volunteers with the standard error of the mean (SEM) in parenthesis. The statistical analysis took the form of a paired Wilcoxon test comparing 0 with all other concentrations of 25(OH)₂D₃. There were no significant differences.

Lymphocyte Activation induced by PHA, Con A and
PWM in the presence of bovine (1 - 35) PTH

b (1 - 35) PTH	PHA		ConA		PWM	
	5µg/ml		50µg/ml		0.1µg/ml	
(Molar)	CPM (x 10 ⁻³)	SI (x 10 ⁻³)	CPM (x 10 ⁻³)	SI (x 10 ⁻³)	CPM (x 10 ⁻³)	SI (x 10 ⁻³)
0	27.4 (1.8)	0.38 (0.06)	23.4 (1.5)	0.32 (0.05)	14.5 (1.9)	0.11 (0.02)
10 ⁻¹³	25.8 (1.8)	0.38 (0.05)	22.1 (1.4)	0.32 (0.04)	13.8 (1.9)	0.12 (0.03)
10 ⁻¹²	25.8 (1.5)	0.33 (0.06)	21.4 (1.5)	0.28 (0.05)	13.1 (1.6)	0.09 (0.02)
10 ⁻¹¹	25.4 (1.4)	0.36 (0.08)	21.5 (1.3)	0.30 (0.06)	12.7 (1.5)	0.10 (0.03)
10 ⁻¹⁰	25.0 (1.6)	0.30 (0.07)	21.8 (1.3)	0.25 (0.05)	12.7 (1.6)	0.07 (0.02)
10 ⁻⁹	25.2 (1.4)	0.34 (0.08)	22.9 (1.6)	0.29 (0.06)	13.7 (1.8)	0.10 (0.02)
10 ⁻⁸	27.3 (2.0)	0.39 (0.07)	22.9 (1.4)	0.30 (0.03)	15.4 (2.0)	0.11 (0.02)
10 ⁻⁷	30.7 (1.7)	0.47 (0.07)	25.6 (2.0)	0.37 (0.05)	15.8 (2.2)	0.13 (0.03)

TABLE 3.4.4.

The table presents the data from an experiment designed to show the effect of bovine (1 - 35) parathyroid hormone on either PHA, Con A or PWM induced lymphocyte activation. The data were presented as both the cpm of tritiated thymidine incorporated and the stimulation index at the various concentrations of b (1 - 35) PTH. The data were the mean of 10 normal volunteers with the standard error of the mean (SEM) in parenthesis. The data were subjected to a paired Wilcoxon test comparing 0 to all other concentrations of b (1 - 35) PTH. There were no significant differences.

Lymphocyte Activation induced by PHA, Con A and
PWM in the presence of salmon calcitonin

s CT	PHA 5µg/ml		ConA 50µg/ml		PWM 0.1µg/ml	
(Molar)	CPM (x 10 ⁻³)	SI (x 10 ⁻³)	CPM (x 10 ⁻³)	SI (x 10 ⁻³)	CPM (x 10 ⁻³)	SI (x 10 ⁻³)
0	33.6 (2.1)	0.57 (0.16)	26.1 (1.7)	0.44 (0.11)	14.6 (1.6)	0.12 (0.03)
10 ⁻¹³	31.1 (2.2)	0.65 (0.13)	24.8 (2.1)	0.49 (0.10)	14.3 (1.7)	0.14 (0.03)
10 ⁻¹²	31.5 (1.8)	0.61 (0.10)	24.4 (1.8)	0.47 (0.07)	14.0 (1.5)	0.14 (0.03)
10 ⁻¹¹	31.7 (2.0)	0.80 (0.20)	24.4 (1.5)	0.60 (0.14)	14.2 (1.5)	0.19 (0.04)
10 ⁻¹⁰	31.3 (2.3)	0.46 (0.12)	25.7 (1.7)	0.38 (0.09)	14.5 (1.5)	0.10 (0.02)
10 ⁻⁹	32.9 (2.1)	0.50 (0.12)	24.7 (1.5)	0.39 (0.09)	14.1 (1.6)	0.11 (0.03)
10 ⁻⁸	34.7 (2.0)	0.76 (0.14)	25.3 (1.6)	0.56 (0.11)	15.0 (1.8)	0.18 (0.04)
10 ⁻⁷	43.0 (4.4)	0.75 (0.13)	28.1 (1.7)	0.48 (0.09)	17.7 (2.6)	0.16 (0.04)

TABLE 3.4.5.

The table presents the data from an experiment designed to show the effect of salmon calcitonin on either PHA, Con A or PWM induced lymphocyte activation. The data were presented as both the cpm of tritiated thymidine incorporated and the stimulation index at the various concentrations of sCT. The data were the mean of 10 normal volunteers with the standard error of the mean (SEM) in parenthesis. The data were statistically analysed using a paired Wilcoxon test comparing 0 with all other concentrations of sCT. There were no significant differences.

Tables 3.4.4. and 3.4.5. depict the effects of PTH and CT on lymphocyte activation. Physiological concentrations in serum are between 10^{-12} M and 5×10^{-12} M for PTH and between 0 and 1.3×10^{-11} M for CT. Again there were no significant differences throughout the range studied as far as the lymphocyte activation assay was concerned. It was of interest that both PTH and CT appeared to show the enhancement of lymphocyte activation. Although observed both in terms of cpms and stimulation indices the enhancement was not statistically significant. The enhancement was perhaps more noticeable in lymphocyte activation in the presence of calcitonin.

3.5. SEX HORMONES AND THE IMMUNE SYSTEM.

Clinical observations have detected striking differences between the immune responsiveness of males and that of females (Ahmed, 1985). In general, the humoral and cell mediated immunity displayed by females is superior to their male counterpart. Women are more resistant to a variety of infections (Thompson, 1963, Washburn, 1965 and Goble, 1971) and this may have a bearing on the well known statistic that they live longer. Perhaps as a consequence of this more sensitive immune system, woman also have exaggerated responses to autoantigens and hence are more susceptible to autoimmune diseases. A striking example of this is seen in the ratio of females to males with systemic lupus erythematosus (SLE) - about 9 : 1 (Dubois, 1974, Kornreich, 1976). Pregnancy is a time of considerable endocrine changes and as such can dramatically alter the course of concurrent autoimmune diseases. The alterations in the circulating levels of sex hormones may account in part for improvement in for example the clinical features of rheumatoid arthritis in pregnancy (Hench, 1938). Pregnancy has also been shown to depress cell mediated and mitogen responses (Purtillo, 1972).

There is more evidence for a role of sex hormones in autoimmune disease if one looks at what happens when oral contraceptives are administered to patients with autoimmune diseases. The disease course may be altered by such regimes : for example, administration of oestrogen - containing contraceptives to lupus patients is associated with exacerbation of the disease (Ahmed, 1985). Oral contraceptives have, however, also been shown to protect against rheumatoid arthritis (Wingrave, 1978).

All the evidence, however, does not come from females: orchiectomy in males delays thymic involution and causes a thymic hypertrophy. It has also been reported that orchiectomy increased protection against viral and fungal and bacterial and parasitic infections (Cohn, 1979). Clearly the immune system does not function in isolation but is intricately involved with other physiological systems in particular the endocrine system. In view of the involvement of oestrogen in bone metabolism it seemed important to investigate the relationship between the sex hormones, the calciotropic hormones and the immune system.

3.5.1. A comparison of $1,25(\text{OH})_2\text{D}_3$ and the various sex hormones as they effect lymphocyte activation.

In section 3.4., a significant inhibition of PHA induced proliferation was observed when cells were cultured in the presence of $1,25(\text{OH})_2\text{D}_3$. Evidence exists for a similar effect when cells are stimulated in the presence of various sex hormones (Holdstock, 1982). The aim of this work was to compare such inhibition in the presence of $1,25(\text{OH})_2\text{D}_3$ with the inhibition in the presence of various sex hormones. The sex hormones studied were oestrogen, progesterone and dihydrotestosterone (DHT).

PBMCs were obtained from nine healthy individuals by density gradient centrifugation (section 2.2.1.). The cells were set up as for lymphocyte activation assay (section 2.7.) in the presence of PHA (5 $\mu\text{g}/\text{ml}$) and a range of concentrations for $1,25(\text{OH})_2\text{D}_3$, oestrogen, progesterone and dihydrotestosterone (10^{-13} M - 10^{-7} M). Each well contains 10^5 cells, PHA at 5 $\mu\text{g}/\text{ml}$ and a specific concentration of either $1,25(\text{OH})_2\text{D}_3$, oestrogen, progesterone and dihydrotestosterone all in a final volume of 200 μl . Each test was again carried out in triplicate. The tests were incubated at 37 °C in a humidified atmosphere of 5 % CO_2/Air for 68 hours. At 68 hours, 1 μCi of tritiated thymidine was added to each test well and the tests were incubated for a further four hours. The test wells were harvested and counted (section 2.7.4.). The results were expressed as counts per minute (cpm) of tritiated thymidine incorporated.

3.5.2. Inhibitory effects of 1,25(OH)₂D₃ and of the sex hormones.

The effects of 1,25(OH)₂D₃ and the three sex hormones (oestrogen, progesterone and dihydrotestosterone) on PHA induced lymphocyte activation are documented in table 3.5.1. (the results are expressed as the mean with the standard error of the mean (SEM) in parenthesis). The inhibition of PHA induced lymphocyte activation by 1,25(OH)₂D₃ achieves significance at concentrations as low as 10⁻¹² M and was sustained throughout the test range. The sex hormones, on the other hand, exhibit an entirely different pattern of inhibition. All three sex hormones show a distinct U-shaped inhibition with peak inhibition occurring at 10⁻¹¹ M (figure 3.5.1.). Of the sex hormones tested, progesterone was the most effective inhibitor of PHA induced lymphocyte activation.

3.5.3 1,25(OH)₂D₃ - The odd one out.

1,25(OH)₂D₃ was a more potent inhibitor of PHA induced lymphocyte activation than either of the sex hormones tested. Progesterone was the most effective sex hormone when it comes to inhibition of PHA induced lymphocyte activation. Progesterone matches 1,25(OH)₂D₃ up to 10⁻¹¹ M as far as inhibition was concerned. The sex hormones exhibit a characteristic U-shaped curve throughout the range of concentrations. A possible explanation for this phenomenon i.e. the abolition of inhibition at concentrations higher than 10⁻¹⁰ M could be down regulation of the sex hormone receptor. It could be that this phenomenon was not apparent in 1,25(OH)₂D₃ because at the higher concentrations a second mechanism was involved in 1,25(OH)₂D₃ or alternatively 1,25(OH)₂D₃ was acting through a completely different mechanism from the sex hormones.

A comparison of the effects of 1,25 dihydroxyvitamin D₃, oestrogen,
progesterone and dihydrotestosterone on PHA - induced lymphocyte
activation

Steroid Hormone Concentration (Molar)	1,25(OH) ₂ D ₃ CPM (x 10 ⁻³)	Oestrogen CPM (x 10 ⁻³)	Progesterone CPM (x 10 ⁻³)	Dihydrotestosterone CPM (x 10 ⁻³)
0	17.4 (1.6)	15.7 (1.5)	15.6 (1.6)	15.4 (1.5)
10 ⁻¹³	15.3 (1.3)	14.8* (1.4)	14.2 (1.4)	14.4 (1.6)
10 ⁻¹²	14.1* (1.6)	14.4 (1.3)	12.6* (1.5)	14.1 (1.6)
10 ⁻¹¹	13.2* (1.5)	13.9* (1.4)	12.3* (1.4)	13.4* (1.5)
10 ⁻¹⁰	11.1* (1.4)	14.3* (1.4)	13.1* (1.6)	14.1* (1.7)
10 ⁻⁹	8.4* (1.2)	14.6 (1.3)	13.6* (1.5)	14.4* (1.7)
10 ⁻⁸	7.4* (1.4)	14.8* (1.5)	14.2 (1.2)	15.1 (1.5)
10 ⁻⁷	8.7* (1.5)	15.8 (1.5)	15.5 (1.6)	15.4 (1.5)

TABLE 3.5.1.

The table presents the data from an experiment that compares the effects of the various sex hormones (oestrogen, progesterone and dihydrotestosterone) with 1,25(OH)₂D₃ with regard to PHA induced lymphocyte activation. The data is presented as the mean of 9 normal individuals with the standard error of the mean (SEM) in parenthesis. The data is the cpm of tritiated thymidine incorporated. The data were statistically analysed using a paired Wilcoxon test (* p < 0.05) comparing 0 and all other concentrations for each substance under test.

A comparison of the effects of 1,25 dihydroxyvitamin
and the various sex hormones on PHA - induced
lymphocyte activation

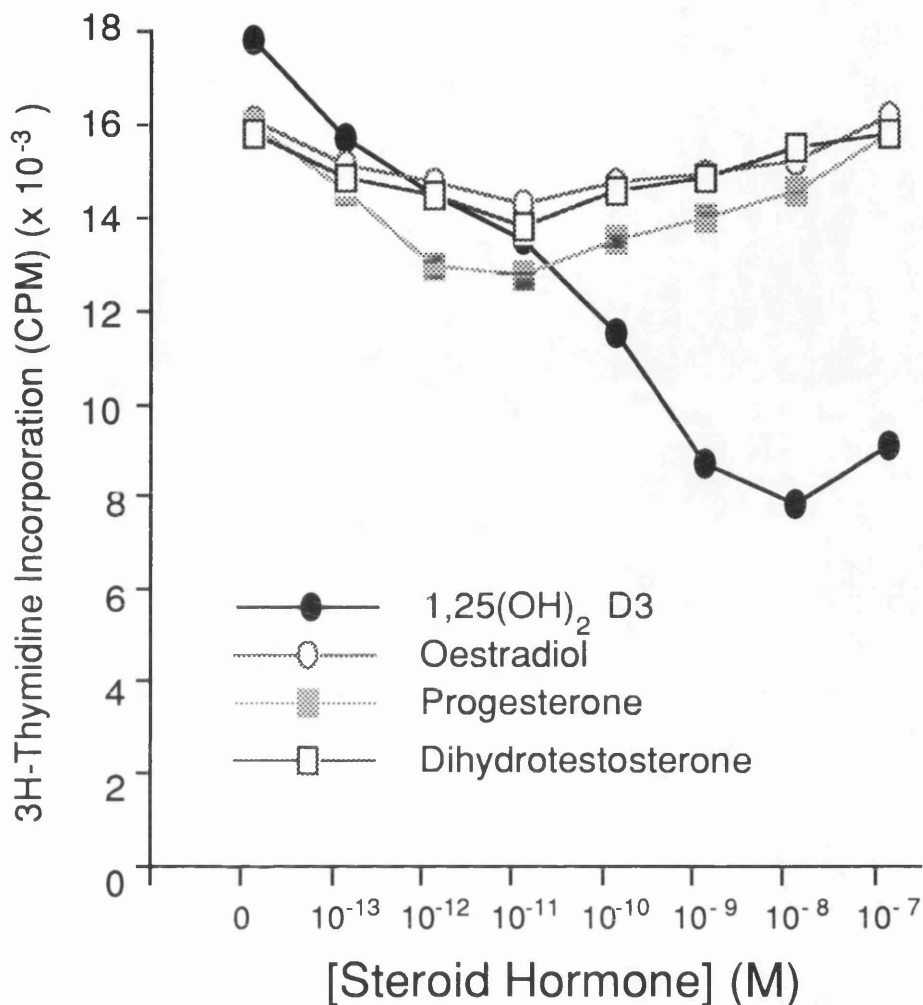


FIGURE 3.5.1.

The figure represents the data from table 3.5.1. in the form of a graph. The data points for each hormone in the figure is presented as the mean of 9 normal individuals. The data points are the cpm of tritiated thymidine incorporated.

3.6. INHIBITION BY 1,25(OH)₂D₃ - A KINETIC EFFECT.

The inhibition of PHA induced lymphocyte activation by 1,25(OH)₂D₃ was now established, it was important to ascertain the mechanism of this effect. It was possible that 1,25(OH)₂D₃ exerted its effect on lymphocyte activation by altering the kinetics of the response of lymphocytes to mitogen. If this was the case then 1,25(OH)₂D₃ would alter the time at which maximum activation would occur. This of course could in theory be earlier or later. However, it is easy to envisage a situation where 1,25(OH)₂D₃ slowed down the rate of lymphocyte activation thereby delaying the maximum activation and creating the impression of inhibition at the time when the assay was harvested i.e. 72 hours. The aim of this next experiment was to establish whether 1,25(OH)₂D₃ altered the kinetics of the lymphocyte activation response.

3.6.1. The time course of 1,25(OH)₂D₃ inhibition in PHA induced PBMC's.

PBMCs were obtained from four normal individuals by density gradient centrifugation (section 2.2.1.). The cells were set up for a lymphocyte activation assay (section 2.7.). The cells were cultured in the presence of PHA (5 µg/ml) and a range of 1,25(OH)₂D₃ concentrations (10⁻¹³ M - 10⁻⁸ M) (section 2.5.). The cultures were incubated at 37 °C in a humidified atmosphere of 5 % CO₂/Air. The cultures were harvested at either 24, 48, 72, 120 or 168 hours. Four hours prior to harvesting the cultures 1 µCi of tritiated thymidine was added to each well. The cultures were harvested on a cell harvester and the results were expressed as counts per minute of tritiated thymidine incorporated.

A study of the kinetics of the effect of
1,25 dihydroxyvitamin D₃ on PHA induced
lymphocyte activation

3H-Thymidine incorporation (CPM) (x 10 ⁻³)					
Length of Incubation (hours)					
1,25(OH) ₂ D ₃ Molar	24	48	72	120	168
0	0.1 (0.02)	12.7 (1.4)	36.3 (1.6)	18.8 (2.5)	4.2 (1.0)
10 ⁻¹³	0.08 (0.01)	12.3 (1.1)	39.3 (1.5)	20.4 (2.9)	4.5 (1.0)
10 ⁻¹²	0.07 (0.01)	12.1 (1.0)	36.6 (2.0)	20.2 (2.9)	4.6 (1.0)
10 ⁻¹¹	0.07 (0.01)	11.8 (0.9)	35.1 (1.9)	20.5 (4.6)	4.3 (1.1)
10 ⁻¹⁰	0.06 (0.01)	10.8 (0.8)	30.4 (2.5)	10.1 (2.7)	4.1 (1.4)
10 ⁻⁹	0.06 (0.01)	10.4 (0.6)	20.8 (3.1)	4.2 (0.9)	1.9 (0.4)
10 ⁻⁸	0.06 (0.01)	9.9 (0.5)	15.9 (2.2)	3.0 (0.7)	1.7 (0.3)

TABLE 3.6.1.

The table presents the data from an experiment that investigates the kinetics of the 1,25(OH)₂D₃ inhibition of the PHA induced lymphocyte activation. The data are presented as the mean of 4 normal individuals with the standard error of the mean (SEM) in parenthesis. The numbers are the cpm of tritiated thymidine incorporated. Statistics were inappropriate.

3.6.2. 1,25(OH)₂D₃ - Not a kinetic effect.

The question of 1,25(OH)₂D₃ exerting a kinetic effect on lymphocyte activation is dealt with in table 3.6.1.. The data reveals that the inhibition of lymphocyte activation by 1,25(OH)₂D₃ was present at all the time points assayed. At longer time points i.e. greater than 72 hours interpretation of the results are more complicated as the cells will not be as viable at these times. However, the trend of inhibition remains the same and this argues against the proposed kinetic effect. 1,25(OH)₂D₃ therefore specifically inhibits lymphocyte activation and this does not occur through changes in the kinetics of lymphocyte activation. The data also confirm that peak activation occurs at 72 hours.

3.7. 1,25(OH)₂D₃ : A DIRECT ACTION ON LYMPHOCYTE ACTIVATION ?

It is possible that 1,25(OH)₂D₃ was acting directly on the lymphocyte activation process. 1,25(OH)₂D₃ could quite conceivably act at the level of transduction of the mitogenic signal to inhibit activation directly. A second possibility is that 1,25(OH)₂D₃ was having a nonspecific effect on the tritiated thymidine incorporation. The following experiment was set up to test both of these possibilities.

3.7.1. Does 1,25(OH)₂D₃ interfere with PHA induced activation ?

PBMCs from four normal individuals were obtained by density gradient centrifugation (section 2.1.1.). The cells were set up in a lymphocyte activation assay (section 2.7.). The cells were cultured in the presence of PHA (5 µg/ml). The cultures were incubated at 37 °C in a humidified atmosphere of 5 % CO₂/Air. 1,25(OH)₂D₃ (10⁻¹⁰ M) was added to certain cultures at various time points. Controls received the vehicle alone at these points. The additions were made at 0, 24 and 48 hours. The assays were pulsed at 68 hours with tritiated thymidine and cultured for a further 4 hours. The cells were harvested and counted the results were presented as counts per minute.

The effect of adding 1,25dihydroxyvitamin D3
at various time points

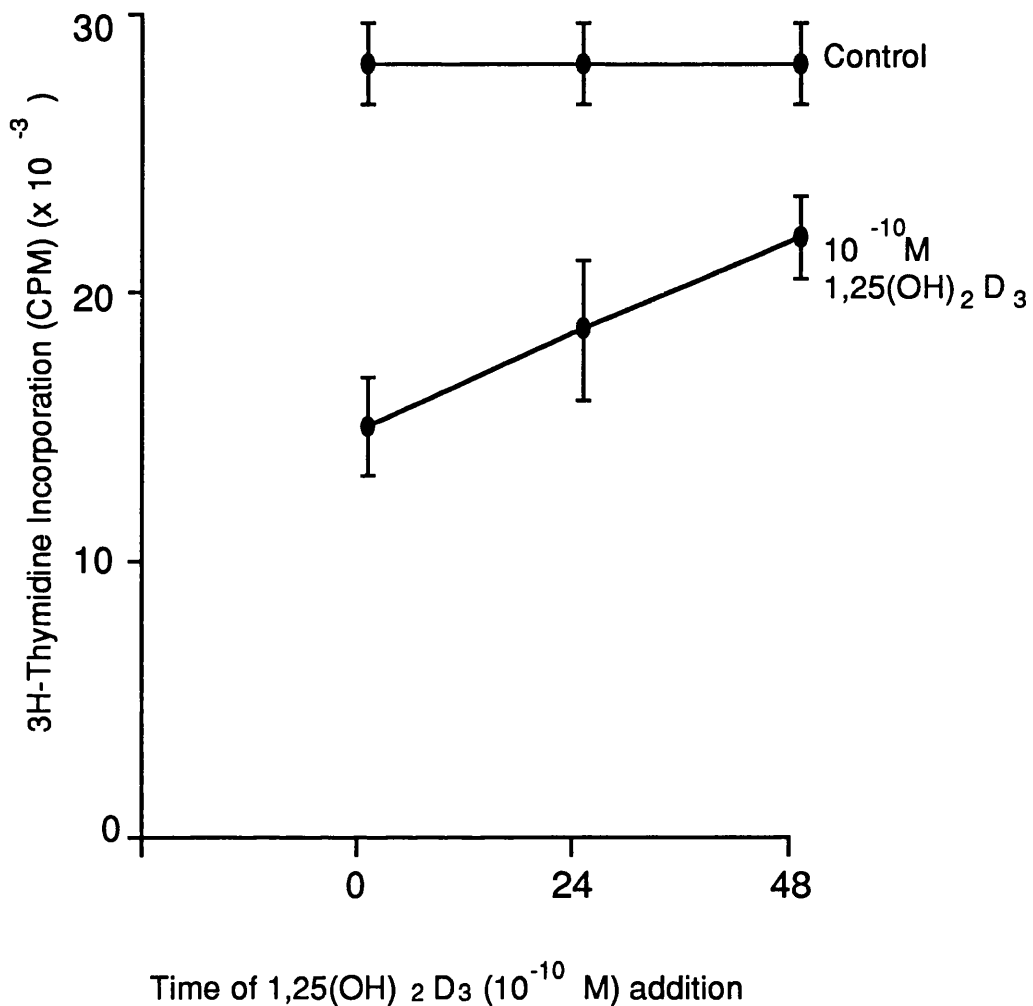


FIGURE 3.7.1.

The figure represents the effect of 1,25 dihydroxyvitamin D3 on PHA induced lymphocyte activation. The 1,25(OH)₂D₃ was added at various time points, at the start of the culture (0 hours) and at 24 and 48 hours. The data are presented as the mean of four individuals with the standard error of the mean (SEM) represented as error bars. Statistical tests were inappropriate.

3.7.2. 1,25(OH)₂D₃ - A genuine inhibitor.

The effect of adding 10^{-10} M 1,25(OH)₂D₃ at different times is represented in figure 3.7.1.. It was obvious that the maximal inhibitory effect was obtained when 1,25(OH)₂D₃ was added at the start of the culture. Addition of 1,25(OH)₂D₃ at 24 and 48 hours resulted in an inhibition but this inhibition was not as pronounced. The data argues against the 1,25(OH)₂D₃ interfering with the activation process as inhibition can be observed when the 1,25(OH)₂D₃ was added after the activation cascade was well underway. The fact that the inhibition was not constant regardless of the addition time of the 1,25(OH)₂D₃, - indeed there was a graduated response - indicates that the inhibition effect by 1,25(OH)₂D₃ is not due to a nonspecific effect on tritiated thymidine uptake.

3.8. DOES 1,25(OH)₂D₃ ALTER VIABILITY ?

The final hurdle to establish that the inhibition of PHA induced lymphocyte activation by 1,25(OH)₂D₃ was a genuine effect was to investigate the viability of the cells in culture. Could the 1,25(OH)₂D₃ related inhibition be due to the 1,25(OH)₂D₃ causing cell death at the higher concentrations of this vitamin D metabolite?

3.8.1. Cell viability in the presence of 1,25(OH)₂D₃.

PBMCs were obtained from eight normal healthy volunteers by density gradient centrifugation (section 2.1.1.). The cells were set up in a lymphocyte activation assay. The cells were cultured in the presence of PHA (5 µg/ml) and in the presence of a range of 1,25(OH)₂D₃ concentrations (10⁻¹³ M - 10⁻⁷ M). The viability of the cells were assessed by trypan blue exclusion (section 2.2.4.) at the beginning of the culture. The cells were cultured at 37 °C in a humidified atmosphere of 5 % CO₂/Air for 72 hours. At 72 hours the viability was again assessed by trypan blue exclusion. Briefly, 10 µl of cells were added to 10 µl of Trypan blue and the number of viable cells were counted along with the total number of cells. The percentage viability was obtained for each 1,25(OH)₂D₃ concentration.

3.8.2. 1,25(OH)₂D₃ - No profound effect on viability.

Table 3.8.1. describes the viability of the PBMCs prior to culture in the presence of 1,25(OH)₂D₃ and the viability of the same PBMCs after 72 hours in the presence of 1,25(OH)₂D₃. Clearly, there was no difference in the viability of the cells at the end of the culture period regardless of the 1,25(OH)₂D₃ concentration. Although the viability has gone down from the initial value at the start of the culture this was as expected. The results would indicate that the inhibition of mitogen induced lymphocyte activation by 1,25(OH)₂D₃ was not secondary to a cytotoxic effect.

The viability of cells cultured in the
presence of 1,25 dihydroxyvitamin D3

1,25(OH) ₂ D ₃ (Molar)	Viability 0 hours Percentage(%)	Viability 72 hours Percentage(%)
0	98.6 (1.0)	79.0 (9.3)
10 ⁻¹³	98.6 (1.0)	76.7 (10.9)
10 ⁻¹²	98.6 (1.0)	83.1 (1.5)
10 ⁻¹¹	98.6 (1.0)	76.7 (12.7)
10 ⁻¹⁰	98.6 (1.0)	80.8 (13.1)
10 ⁻⁹	98.6 (1.0)	81.7 (8.7)
10 ⁻⁸	98.6 (1.0)	88.2 (9.6)
10 ⁻⁷	98.6 (1.0)	79.7 (6.7)

TABLE 3.8.1.

The table presents the data from an experiment to assess the effect of 1,25 dihydroxyvitamin D₃ on the viability of PBMCs cultured for 72 hours. The data was presented as the percentage viability as measured in a trypan blue exclusion experiment. The data was obtained from 8 normal individuals and the mean was presented with the standard error of the mean in parenthesis. Statistics were carried out between 0 and all other 1,25(OH)₂D₃ concentrations using a paired Wilcoxon test and no significant differences were obtained between the different 1,25 dihydroxyvitamin D₃ concentrations at either time point.

3.9. 1,25(OH)₂D₃ THE EFFECTIVE INHIBITOR OF LYMPHOCYTE ACTIVATION.

Both PTH and CT failed significantly to alter lymphocyte activation stimulated by the mitogens, PHA, Con A and PWM. This was observed despite the fact that lymphocytes possess the appropriate receptors. Careful examination of the data reveals a trend of enhanced lymphocyte activation although this never achieved significance. It may be that the receptors for PTH and CT are not important to the mature lymphocyte participating in the immune system, but may be important earlier on in the cells development. It is known that CT and PTH can influence the proliferation of immature T-cells.

The D₃ metabolites, 24,25(OH)₂D₃ and 25(OH)D₃, also failed to significantly alter lymphocyte activation by the three mitogens. The active metabolite of vitamin D₃, 1,25(OH)₂D₃ significantly inhibited lymphocyte activation by all three mitogens. The normal physiological range of 1,25(OH)₂D₃ in normal serum occurs between 10⁻¹¹ M and 10⁻¹⁰M. The inhibition of PHA induced lymphocyte activation by 1,25(OH)₂D₃ becomes significant at 10⁻¹⁰ M. These observations would argue in favour for a role for 1,25(OH)₂D₃ in immunoregulation. Con A and PWM stimulated lymphocyte activation achieve significance at 10⁻⁸ M and 10⁻⁹ M respectively (Smith, 1990).

In order to fully appreciate the significance of the inhibition of lymphocyte activation by 1,25(OH)₂D₃ the percentage inhibition was calculated. The data presented in table 3.9.1. represents the percentage inhibition by 1,25(OH)₂D₃ for all three mitogens. The percentage inhibition was calculated using the following formulae;

$$\text{percentage inhibition (\%)} = 1 - \frac{\text{cpm}(\text{experimental culture})}{\text{cpm}(\text{control culture})} \times 100$$

Inhibition of PHA -, Con A - and PWM - induced lymphocyte activation
in the presence of 1,25 dihydroxyvitamin D3 expressed as a percentage

1,25(OH) ₂ D ₃ (Molar)	PHA Inhibition (%)	ConA Inhibition (%)	PWM Inhibition (%)
0	0	0	0
10 ⁻¹³	-2.4 (12.3)	-6.4 (12.2)	-2.3 (10.2)
10 ⁻¹²	-2.6 (10.6)	1.8 (14.5)	-4.4 (13.3)
10 ⁻¹¹	-0.6 (14.5)	-1.4 (14.1)	0.4 (10.1)
10 ⁻¹⁰	19.1* (13.9)	1.0 (11.8)	8.7* (8.4)
10 ⁻⁹	49.8* (9.9)	17.9* (22.0)	12.6* (12.9)
10 ⁻⁸	54.9* (5.6)	21.9* (13.5)	10.5* (15.3)
10 ⁻⁷	48.1* (9.0)	22.4* (14.5)	6.5* (11.3)

TABLE 3.9.1.

The table presents the data from the experiment that demonstrated the inhibition of PHA, Con A and PWM induced lymphocyte activation by 1,25 dihydroxyvitamin D3 (see section 3.4.2.) expressed as the percentage inhibition. The formulae is presented in the text. The data was obtained from 10 normal individuals and the mean and the standard error of the mean are presented in parenthesis. Statistics were carried using a paired Wilcoxon test (* p < 0.05) comparing 0 with all other concentrations of each substance under review.

Inhibition of PHA - induced lymphocyte activation in the
presence of 1,25 dihydroxyvitamin D3, oestrogen, progesterone
and dihydroxytestosterone

Steroid Hormone Concentration (Molar)	1,25(OH) ₂ D ₃ % inhibition	Oestrogen % inhibition	Progesterone % inhibition	Dihydroxytestosterone % inhibition
0	0	0	0	0
10 ⁻¹³	12.1	5.7	9.0	6.5
10 ⁻¹²	19.0	8.3	19.2	8.4
10 ⁻¹¹	24.1	11.5	21.2	13.0
10 ⁻¹⁰	36.2	8.9	16.0	8.4
10 ⁻⁹	51.7	7.0	12.8	6.5
10 ⁻⁸	57.5	5.7	9.0	1.9
10 ⁻⁷	50.0	-0.6	0.6	0

TABLE 3.9.2.

The table presents the data from the experiment that demonstrated the inhibition of PHA induced lymphocyte activation by 1,25 dihydroxyvitamin D₃, oestrogen, progesterone or dihydrotestosterone (see section 3.5.2.) expressed as the percentage inhibition. The formulae is presented in the text. The data was obtained from 9 normal individuals and the mean and the standard error of the mean are presented in parenthesis.

It is clear that PHA induced lymphocyte activation is inhibited to a greater extent than either PWM or Con A induced lymphocyte activation. Inhibition of PHA induced lymphocyte activation in excess of 50 % was achieved by $1,25(\text{OH})_2\text{D}_3$ whereas inhibition of lymphocyte activation stimulated with Con A and PWM does not exceed 23 % and 13 % respectively. As discussed previously each of these mitogens stimulate a different population of cells in the lymphocyte activation process. PHA is predominantly a T-helper/inducer (T_H) mitogen, it is therefore likely that at physiological concentrations of $1,25(\text{OH})_2\text{D}_3$ this subset of cells is the main target for the inhibition effect. The significant inhibition observed in Con A and PWM induced lymphocyte activation occurs at 100-fold and 10-fold greater concentrations of the $1,25(\text{OH})_2\text{D}_3$ respectively. This inhibition albeit, a small inhibition, can be explained by minor effects on T_H cells. Con A is predominantly a T-suppressor/cytotoxic (T_S) mitogen, however, for the T_S cells to proliferate requires the activation of a population of T_H . PWM is a T-cell dependent B-cell mitogen, and the T-cell on which it is dependent is a T_H cell. This T_H cell allows the B-cell to proliferate. It is conceivable that an inhibition of this T_H cell by $1,25(\text{OH})_2\text{D}_3$ causes the observed inhibition. The T_H cells secrete the cytokine interleukin-2, which is by definition the T-cell growth factor (TCGF).

The sex hormones, oestrogen, progesterone and dihydrotestosterone have also been demonstrated to have some inhibitory effect on PHA induced lymphocyte activation. In many ways the inhibition by these hormones is similar to the inhibition by $1,25(\text{OH})_2\text{D}_3$. Table 3.9.2. expresses the data in the form of a percentage inhibition, which was calculated using the same formulae as was used for table 3.9.1.. In the case of $1,25(\text{OH})_2\text{D}_3$, inhibition of about 60 % was achieved whereas oestrogen, progesterone and dihydrotestosterone produce maximum inhibition of 11.5 %, 21.2 % and 13.0 % respectively. The maximum inhibition occurs at 10^{-11} M and could therefore be important. At 10^{-7} M, the lymphocyte activation is back to control levels in the presence of the sex hormones. The characteristic U-shaped curve is perhaps a consequence of down regulation of their respective hormone receptors.

CHAPTER 4.

INTERLEUKIN -2 (IL-2) ACTIVITY - THE EFFECT OF CALCITROPIC AND SEX HORMONES.

4.1. INTERLEUKIN - 2 (IL-2).

Interleukin-2 (IL-2) was the first immune hormone to be discovered and characterised (Robb, 1981). It has a crucial role to play in the generation of a successful immune response and an understanding of its role has done much to explain the unique properties of the immune system. IL-2 was originally called T-cell growth factor (TCGF) and this name adequately reflected its principal function. IL-2 functions to promote the proliferation of T-cells that have been triggered by a specific antigen, resulting in a clone of identical cells all of which were specific for a particular antigen. Although T-cells are the main target for IL-2, IL-2 receptors (IL-2R) have been identified on other cell types (Mizel, 1989). As a result of the distribution of these receptors, B-cells, Natural killer cells (NK) and Lymphocyte activated killer cells (LAK) were also responsive to IL-2. It should be noted that resting T-cells do not possess the high affinity IL-2R, it is only in the presence of an antigen presenting cell (macrophage) and an appropriate antigen that these receptors are expressed (Gillis, 1978).

4.1.1. IL-2 : Its biochemistry.

Biochemical analysis of IL-2 has revealed that it is a glycoprotein which is heterogeneous in respect of size and charge. IL-2 is secreted as a single polypeptide chain with an approximate molecular weight of 15.5 kilodaltons (Kd), with a slightly basic isoelectric point (pI 8.2 after the removal of sialic acid residues) and is hydrophobic in nature (Robb, 1981). The heterogeneity can be explained by variable glycosylation and sialation of a single protein, the single protein being confirmed by monoclonal antibodies.

Molecular biology enabled Taniguchi et al (Taniguchi, 1983) to produce a complementary DNA (cDNA) for IL-2. The cDNA predicted a mature protein of 15,420 daltons with an NH₂ terminal sequence identical to that found from analysis of the purified protein. Also it has been revealed that the primary translational product of IL-2 messenger RNA (mRNA) contains the classical hydrophobic signal sequence suggesting that IL-2 is released by the pathway associated with all other secretory proteins (Mizel, 1989). The genomic organisation of the IL-2 gene is such that IL-2 is encoded by four exons separated by one short and two long introns (Fujita, 1983). The set-up is similar to other cytokines which points to the existence of a common genetic progenitor.

4.1.2. Interleukin-2 : The pathway of discovery.

The major breakthrough in immunology that facilitated the discovery of IL-2 occurred when it was demonstrated that given the correct stimulus lymphocytes could proliferate (Nowell, 1960). Prior to this it was generally accepted that lymphocytes were incapable of proliferation. The proper stimulus in this case turned out to be the plant lectin phytohaemagglutinin (PHA), however, it soon became apparent that a number of other plant lectins could produce a similar response. It was only a matter of time until the plant lectins were replaced by specific antigens to give the proliferative response thereby validating the proliferative response as a genuine physiological phenomenon. Two groups (Kasakura, 1965; Gordon, 1965) described a soluble mitogenic factor derived from conditioned culture media obtained from mixed lymphocyte culture. At this juncture the scientific community believed that this factor only played a supportive role, perhaps amplifying a process signaled by antigen. Around this time lymphocytes were thought to be the only source of mitogenic factors and so the term lymphokine was coined to describe these factors present in the conditioned media. However, it soon became apparent that other cell types produce other mitogenic factors. In 1976, Morgan et al (Morgan, 1976) demonstrated that normal human T-lymphocytes could be cultured

for extended periods in media that contained conditioned media derived from phytohaemagglutinin stimulated peripheral mononuclear cells. A year later, Gillis and Smith, employed Morgan's observation to produce murine cytolytic T-lymphocyte lines (CTLL) specific for tumour antigens (Gillis, 1977). In 1979 a CTLL was cloned by limiting dilutions to give a monoclonal cytotoxic T-cell line (Baker, 1979). Cloned CTLL cells were the key to unravelling the mystery behind the mitogenic factors. Having obtained a cell line derived from a single cell the question of a heterogenous cell population was solved. The mitogenic factor responsible for long term T-cell growth could now be characterised.

4.2. THE MEASUREMENT OF INTERLEUKIN - 2 (IL-2).

The majority of assays that measure IL-2 are bioassays which assess the proliferative capacity of the lymphokine on responsive cells. Proliferation can be measured in several ways ; the most obvious being to look for an increase in viable cell number. However, the most commonly used method is to assess an increase in DNA synthesis by measuring incorporation of tritiated thymidine ([³H]-thy) into DNA. Incorporation of [³H]-thy reflects accurately the number of cells in the S-phase of the cell cycle. A third alternative is to assess the amount of active mitochondria in the culture. This can be achieved by measuring a colorimetric change brought about when the mitochondrial dehydrogenase enzymes cleave the tetrazolium ring present in M.T.T. (3-(4,5-dimethylthiazol-2yl)-2,5 diphenyl tetrazolium) (Mosmann, 1983). Bioassays require a short term culture in the presence of lymphokine and as a consequence are susceptible to biological variability. These assays also have a problem with specificity. The major advantage of the bioassays is that they are extremely sensitive.

The development of antibodies against IL-2 raised the possibility that immunochemical procedures for measuring IL-2 would be adopted. Enzyme linked immunoabsorbant assays (ELISA) have been developed to measure IL-2 (Gehman, 1984), these assays although reliable are found to be one hundred times less sensitive.

For the most part all bioassays for measuring IL-2 are similar to each other the only variable being the cells that are used as responder cells. The responder cells used in the bioassay fall into three groups;

- (i) Continuous IL-2 dependent cell lines
- (ii) Mitogenic lectin induced IL-2 responsive cells
- (iii) Short-term IL-2 responsive cells

The advantages and disadvantages of each of these groups are discussed in subsequent sections.

4.2.1. Continuous IL-2 dependent cell lines.

The production of IL-2 dependent cell lines is relatively easy if rodent splenocytes are the initial cell source. The splenocytes are stimulated with antigens and/or mitogenic lectins, and cultured long term with regular feeding with IL-2 and/or antigen. After this regime has been established for several weeks, the cells are cloned by the limiting dilution method. The cloned cells are then tested for responsiveness to exogenous IL-2 : the high responding clones are selected and continually grown in culture. The cell lines minimise the variation between assays, and the problems with specificity are limited as the cells have been selected for their ability to respond to IL-2. As a result the assay is specific, reproducible and sensitive, although care has to be taken in the interpretation of the results. The primary disadvantages of these cell lines is that they were difficult to maintain and were difficult to cryopreserve.

The cell line HT-2 are cells produced using just such a protocol. HT-2 cells were derived from murine T-helper cells by Watson (Watson, 1979). These cells require IL-2 for continuous growth and without IL-2 the cells die. The T-cell mitogens Con A and PHA do not stimulate proliferation in long term culture of these cells. HT-2 represents a good cell line to use in an IL-2 bioassay.

4.2.2. Mitogenic lectin induced IL-2 responsive cells.

Stimulation of splenocytes or thymocytes with mitogenic plant lectins induce a subpopulation of lymphocytes to become responsive to IL-2. The responsive cells can then be used in a assay for IL-2. Rodent splenocytes or thymocytes can be easily activated using Con A to provide a cell population that responds to IL-2. The population of cells obtained by this method also respond to a range of stimuli other than IL-2. This problem is particularly relevant when assessing IL-2 in an enriched supernatant which contains residual plant lectin. It is possible to get rid of the offending residual lectin but this is both laborious and time-consuming. It is also possible that the population of cells obtained in this fashion is stimulated by other cytokines (B-cell growth factor (BCGF) and interleukin-1(IL-1)) that are also present in the supernatants.

4.2.3. Short-term IL-2 responsive cells.

It is possible to stimulate T-lymphocytes with lectin or antigen and feed these cells with IL-2, to produce a cell population that is dependent on the lymphokine. The disadvantage of this system is that there is considerable variation depending on the donor cell.

4.3. THE SEARCH FOR A SUITABLE ASSAY.

4.3.1. A comparison of types of bioassays.

A source of purified IL-2 was assessed in three distinct bioassay systems. The bioassay systems employed represent; a continuous IL-2 dependent cell lines, a mitogenic induced IL-2 responsive cells and a short-term IL-2 responsive cells. Serial doubling dilutions of the purified IL-2 were carried out in complete RPMI 1640 and 100 μ l of each dilution added to three 96-well microtiter plate (U-shaped, Bibby Sterlin, U.K.) in triplicate. The responsive cells were prepared as follows;

HT-2 - a continuous IL-2 dependent cell line.

This cell line was maintained in culture as described in section 2.3.1.. Prior to assay the cells were washed three times in RPMI 1640 and resuspended at a cell density of 1.5×10^5 cells/ml in complete RPMI 1640 + 20 % F.C.S..

Con A induced mouse splenocytes - a mitogenic lectin induced IL-2 responsive cells.

An intact spleen was dissected from Balb/c mouse, transferred to complete RPMI 1640 and the splenocytes were gently teased out into the complete RPMI 1640. Tissue debris was allowed to settle to the bottom of a universal and the cells above the debris were transferred to a fresh universal and washed three times in complete RPMI 1640. The cell suspension was seeded in complete RPMI 1640 + 10 % F.C.S. at a cell density of 10^7 cells/ml. The cells were stimulated with 2.5 μ g/ml of Con A. The cells were cultured for three days at 37 °C in a humidified atmosphere of 5 % CO₂/Air. Prior to the assay the cells were washed and resuspended in RPMI 1640 + 20 % F.C.S. at a cell density of 10^6 viable cells/ml.

Con A induced lymphoblasts - a short-term IL-2 responsive cell.

PBMCs were obtained from a normal individual by density gradient centrifugation (section 2.2.1.). The cells were resuspended in complete RPMI 1640 + 5 % F.C.S. at a cell density of 10^6 cells/ml. The cells were stimulated with 5 μ g/ml Con A and cultured at 37 °C in a humidified atmosphere of 5 % CO₂/Air for 3 days. At day 3, the cells were washed and resuspended in complete RPMI 1640 + 5 % F.C.S. at 10^6 cells/ml and cultured under the same conditions for a further 4 days. At day 7 the cells were again washed and resuspended in complete RPMI 1640 + 5 % F.C.S. additionally supplemented with IL-2 containing supernatant (25 %) and cultured for a further 3 days. Prior to the assay the cells were washed and resuspended in RPMI 1640 + 20 % F.C.S. at a cell density of 0.5×10^6 cells/ml.

The assays were started with the addition of the responder cells, 100 μ l of the cell preparations were added to each well containing the IL-2 dilutions. The HT-2 assay was incubated for 24 hours at 37 °C in a humidified atmosphere of 5 % CO₂/Air, 4 hours prior to the end of the assay 1 μ Ci of [³H]-thy was added to each well. The Con A induced mouse splenocytes and the Con A induced lymphoblasts were incubated for 72 hours under the same conditions and 18 hours prior to harvesting 1 μ Ci of [³H]-thy was added to each well. All three IL-2 assays were harvested as in section 2.7.4..

4.3.2. HT-2 - The assay of choice.

Figure 4.3.1. describes the dilution curves for purified IL-2 as assayed in each of the three bioassays. All three bioassays produced similar dilution curves indicating that the bioassays were most likely measuring the same factor, IL-2. When the concentration of the purified IL-2 was calculated using the accepted definition of a unit of IL-2 activity the figure from each of the bioassays were similar. One unit of IL-2 was defined as the amount of IL-2 required to give 50 % maximum activity.

The HT-2 assay was the assay of choice for measuring IL-2 in all subsequent work. The assay had two distinct advantages over the other bioassays ; a short incubation (24 hours) and minimal preparation of responder cells.

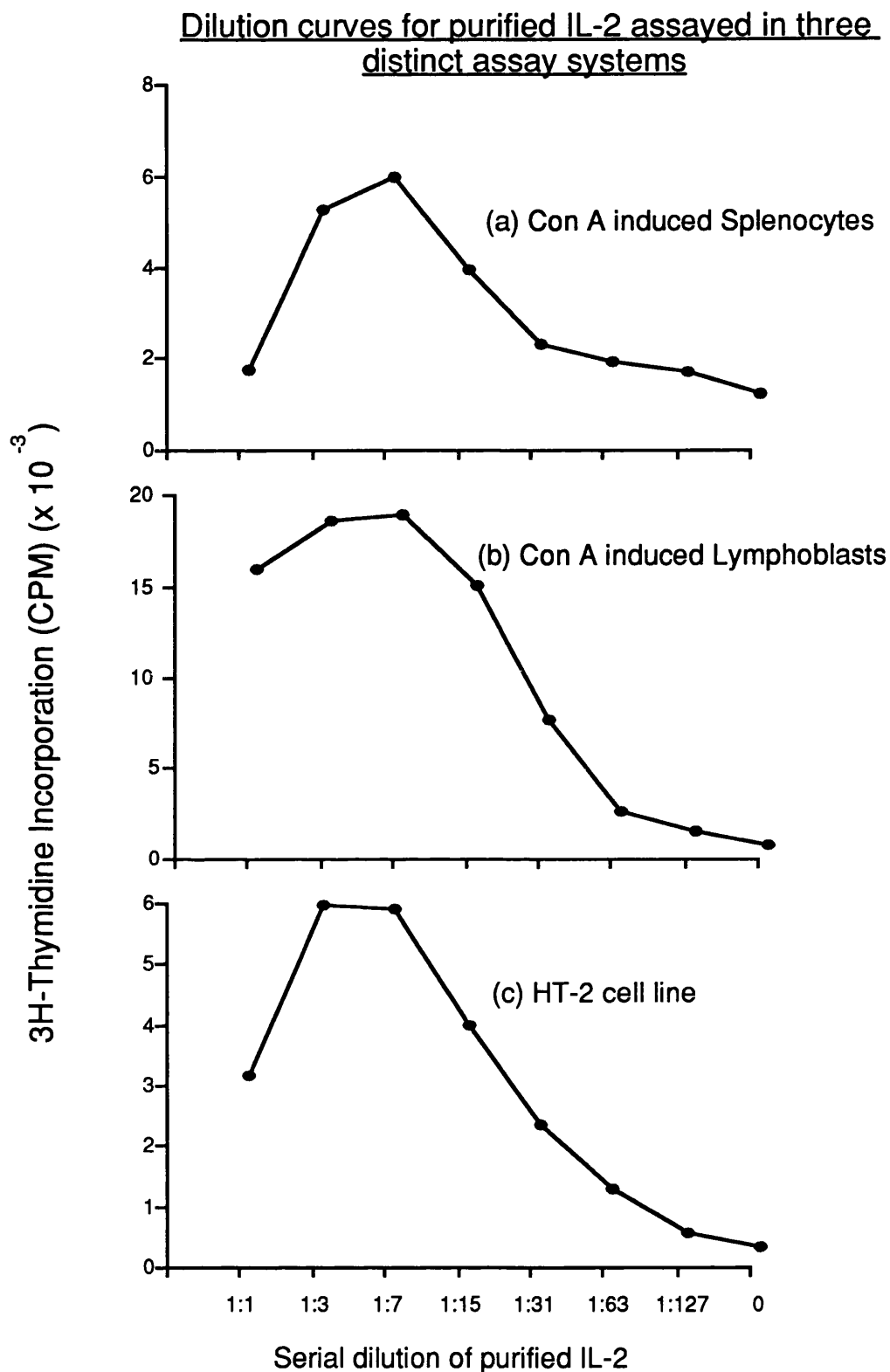


FIGURE 4.3.1.

The figure represents the dilution curve for purified IL-2 in the three assay systems. The data are presented as the cpm of tritiated thymidine incorporated against the dilution of the purified IL-2.

4.4. STANDARDISATION OF INTERLEUKIN - 2 PRODUCTION.

Having made the choice of an assay system for measuring IL-2, the next step was to standardise a method for producing IL-2. Production of IL-2 was achieved by stimulating PBMCs with purified phytohaemagglutinin (Wellcome Grade). The variables in the system were length of stimulation, cell density and PHA concentration.

4.4.1. Optimisation of cell number, PHA concentration and length of incubation.

PBMCs were isolated from eight normal individuals by density gradient centrifugation (section 2.2.1.). The cells were resuspended in complete RPMI 1640 (with HEPES) + 2 % FCS to give the following final concentrations in culture, after addition of PHA, of ;

- (1) 5×10^6 cells/ml
- (2) 2×10^6 cells/ml
- (3) 1×10^6 cells/ml
- (4) 0.5×10^6 cells/ml

PHA was made up in complete RPMI 1640 (without FCS) such that when equal volumes of the PHA solution were added to cells, a final concentration of ;

- (1) 5 µg/ml
- (2) 2 µg/ml
- (3) 1 µg/ml
- (4) 0.5 µg/ml

was achieved. The cultures were incubated at 37 °C in a 5 % CO₂/Air humidified atmosphere for 24 hours or 48 hours. At the end of the incubation period the IL-2 containing supernatants were harvested (section 2.8.1.). The IL-2 present in the supernatants was assessed in the HT-2 bioassay (section 2.8.2.).

4.4.2. Standardisation - The verdict.

The results are presented in figures 4.4.1. and 4.4.2.. Figure 4.4.1. represents the dilution curves for the different cell concentrations stimulated with 2 $\mu\text{g/ml}$ PHA for 48 hours. Figure 4.4.2. portrays the dilution curves for the same cell concentrations stimulated with 1 $\mu\text{g/ml}$ for 48 hours. The cells stimulated with 5 $\mu\text{g/ml}$ PHA gave a mixed response and were not represented ; the cells stimulated with 0.5 $\mu\text{g/ml}$ PHA did not produce detectable amounts of IL-2. The time of incubations was set at 48 hours, as the response at 24 hours was minimal. These data on the whole agree with published findings (Tsoukas, 1984).

Figure 4.4.1. indicates that densities of both 10^6 cells/ml and 0.5×10^6 cells/ml produce similar dilution curves when stimulated with 2 $\mu\text{g/ml}$ PHA. Figure 4.4.2. also indicates that 10^6 cells/ml and 0.5×10^6 cells/ml stimulated with 1 $\mu\text{g/ml}$ produce similar dilution curves. Essentially if the dilution curves were compared then there was no significant difference between the cells stimulated with either 1 $\mu\text{g/ml}$ or 2 $\mu\text{g/ml}$ PHA. So the choice becomes an economic one, i.e. a concentration of 1 $\mu\text{g/ml}$ was chosen for subsequent assays. Comparison of the cell densities reveals a straight choice between 10^6 cells/ml and 0.5×10^6 cells/ml. There was very little between these two cell densities, the cell density of 10^6 cells/ml was selected because the dilution curve was slightly more linear and also the fact that most of the published work has used this cell density.

Dilution curves for IL-2 supernatant
standardisation

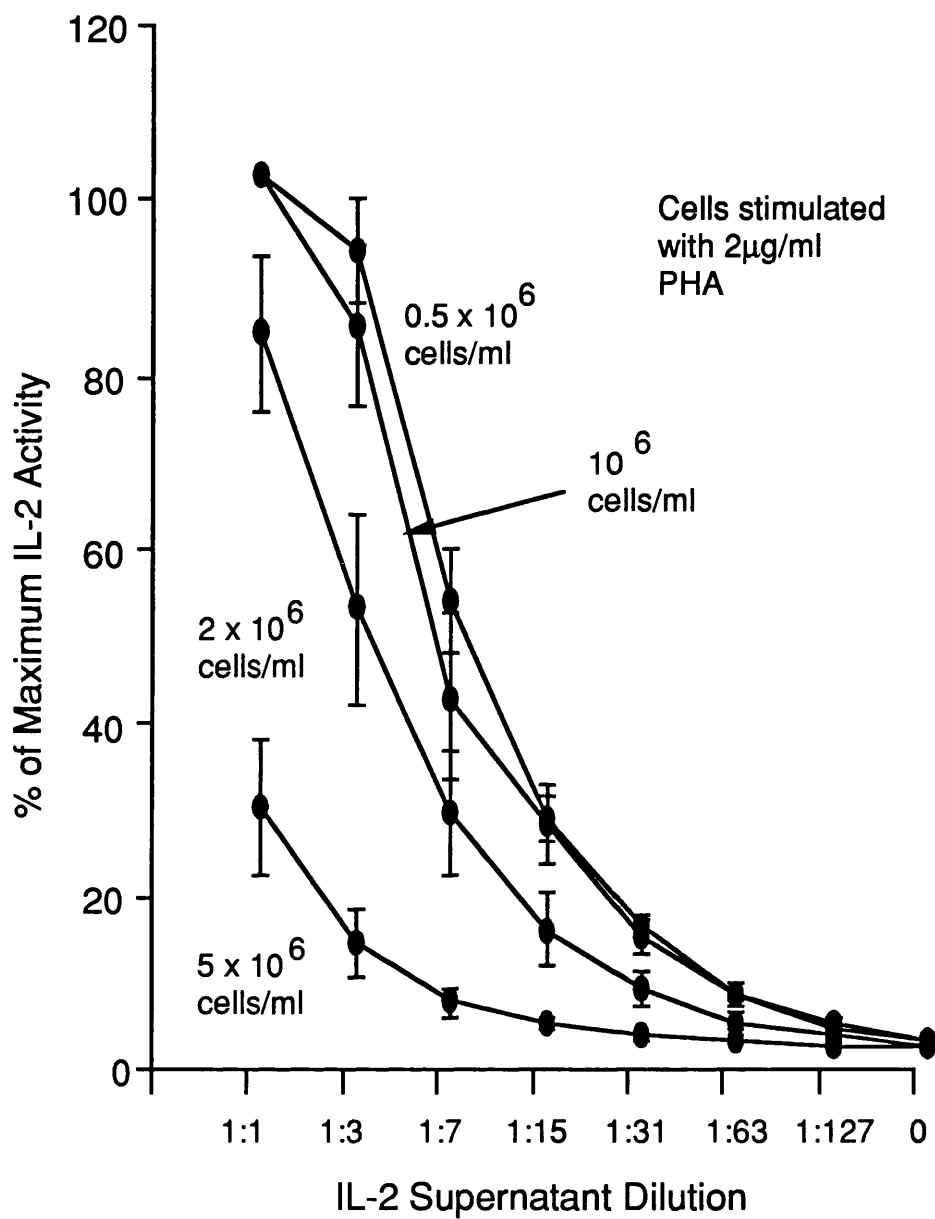


FIGURE 4.4.1.

The figure represents the dilution curve for the standardisation of the cell concentration at 2 µg/ml PHA. The data are the mean of 8 normal individuals and the error bars represent the standard error of the mean (SEM). The incubation time was 48 hours.

Dilution curves for IL-2 supernatant
standardisation

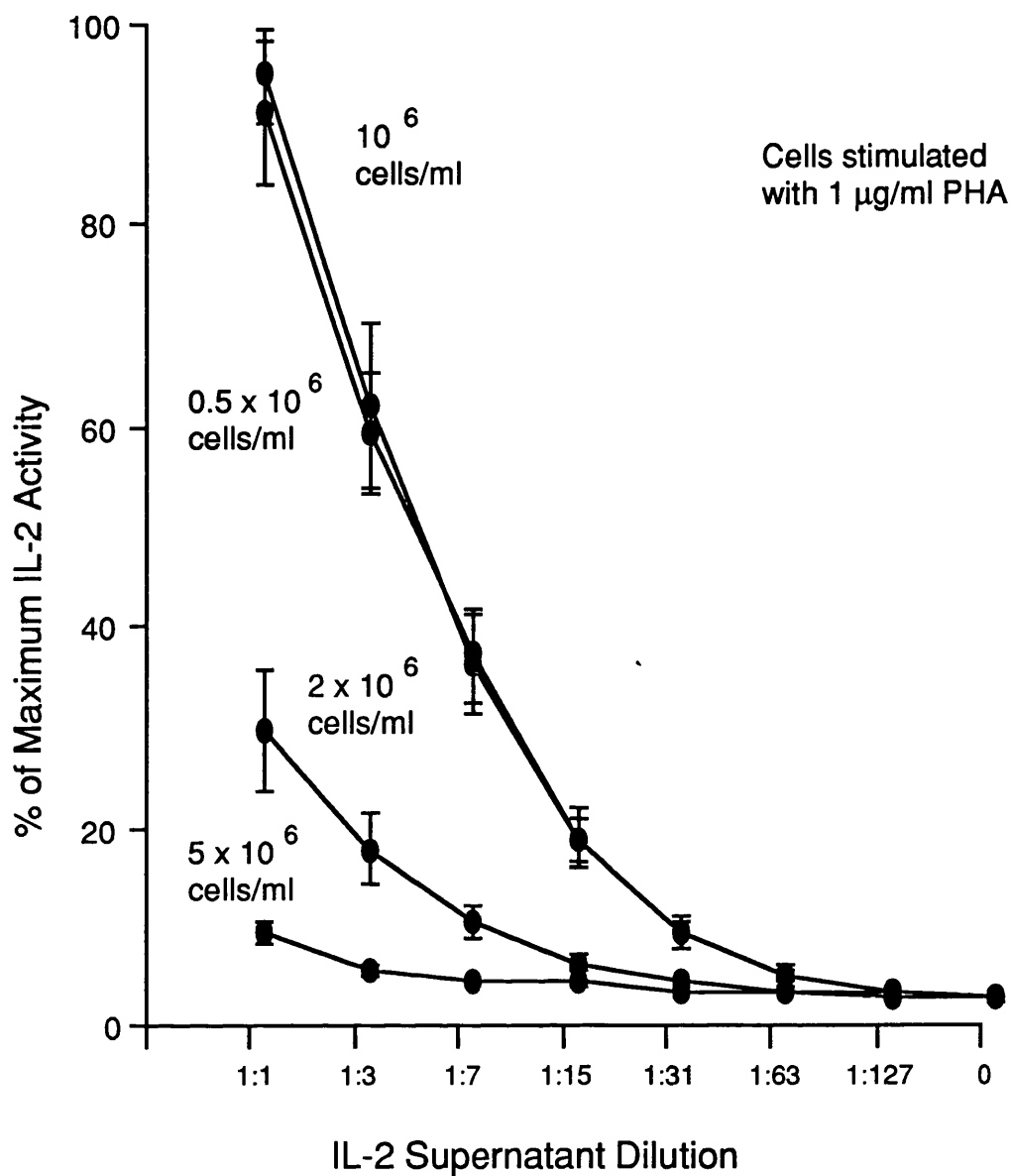


FIGURE 4.4.2.

The figure represents the dilution curves for the standardisation of the cell concentration at 1 µg/ml PHA. The data are the mean of 8 normal individuals and the error bars represent the standard error of the mean (SEM). The incubation time for these supernatants was 48 hours.

4.5. THE EFFECTS OF CALCITROPIC HORMONES ON INTERLEUKIN -2 ACTIVITY.

The results presented in section 3.4. demonstrate that $1,25(\text{OH})_2\text{D}_3$ has a significant effect on lymphocyte transformation and that this effect may be mediated through the T_H subset. IL-2 was the main cytokine produced by this subset of cells so it was decided to investigate the effects of the five calcitrophic hormones on IL-2 activity.

4.5.1. IL-2 activity in the presence of calcitrophic hormones.

PBMCs were isolated from nine normal individuals by density gradient centrifugation (section 2.2.1.). These cells were resuspended at a cell density of 10^6 cells/ml in complete RPMI 1640 + 1% F.C.S. and stimulated with $1\text{ }\mu\text{g/ml}$ PHA (section 2.8.1.) in the presence of a range of concentrations of the calcitrophic hormones (section 2.5.). The cells were cultured for 48 hours, at which time the supernatants were harvested. The amount of IL-2 activity in these supernatants was assessed using the HT-2 bioassay (section 2.8.2.). The results were expressed as units/ml IL-2.

4.5.2. 1,25(OH)₂D₃ - The effective IL-2 inhibitor.

Table 4.5.1. documents the IL-2 activity at the various concentrations of the calcitrophic hormones studied. There was no significant change in the IL-2 activity in the presence of either sCT or b(1-35)PTH. However, at 10⁻⁷ M b(1-35)PTH slightly inhibited IL-2 activity but this did not achieve statistical significance. In the case of the vitamin D₃ metabolites, all three metabolites inhibited IL-2 activity. The active metabolite of vitamin D₃, 1,25(OH)₂D₃, significantly inhibited IL-2 activity at 10⁻¹⁰ M which is within the normal physiological range in serum (10⁻¹¹ M - 10⁻¹⁰M). Both 24,25(OH)₂D₃ and 25(OH)D₃ achieved significance only at 10⁻⁷ M. 24,25(OH)₂D₃ produced a greater inhibition than did 25(OH)D₃ although compared to 1,25(OH)₂D₃ the inhibition was minimal. The magnitude of inhibition fits in nicely with the order of affinity of the 1,25(OH)₂D₃, 24,25(OH)₂D₃ and 25(OH)D₃ for the vitamin D receptor (VDR).

Hormone	Interleukin-2 activity (units/ml)									
	Concentration (M)									
	0	10 ⁻¹³	10 ⁻¹²	10 ⁻¹¹	10 ⁻¹⁰	10 ⁻⁹	10 ⁻⁸	10 ⁻⁷		
1,25(OH) ₂ D ₃	33.4 (10.8)	28.3 (9.7)	31.9 (13.3)	29.7 (11.8)	22.0* (9.5)	14.0* (4.5)	12.4* (4.0)	11.9* (4.0)		
24,25(OH) ₂ D ₃	33.0 (13.1)	29.3 (9.3)	32.0 (11.8)	28.9 (10.2)	33.0 (11.2)	30.7 (10.9)	33.8 (11.7)	15.8* (6.6)		
25(OH)D ₃	29.4 (10.5)	29.0 (9.6)	34.0 (10.8)	34.0 (11.5)	36.2 (12.5)	30.1 (11.4)	29.9 (11.3)	19.5* (7.9)		
b(1-35)PTH	29.1 (10.5)	23.4 (8.5)	32.0 (11.6)	27.2 (10.7)	29.6 (11.2)	27.4 (10.3)	25.5 (9.5)	20.8 (7.3)		
sCT	22.1 (6.2)	20.5 (6.5)	22.7 (7.8)	20.8 (7.0)	21.2 (6.4)	19.9 (6.1)	21.5 (6.4)	23.1 (7.9)		

TABLE 4.5.1.

The table presents the IL-2 activity present in supernatants produced in the presence of the five calcitrophic hormones (1,25(OH)₂D₃, 24,25(OH)₂D₃, 25(OH)D₃, b (1 -35) PTH and sCT). The IL-2 activity is presented as units/ml. The data are the mean of 9 normal individuals. The standard error of the means are presented in parenthesis. Statistics in the form of a paired Wilcoxon test were carried out (* p < 0.05) comparing 0 with all other concentrations of the substance under review.

The effect of 1,25 dihydroxyvitamin D₃
on IL-2 activity

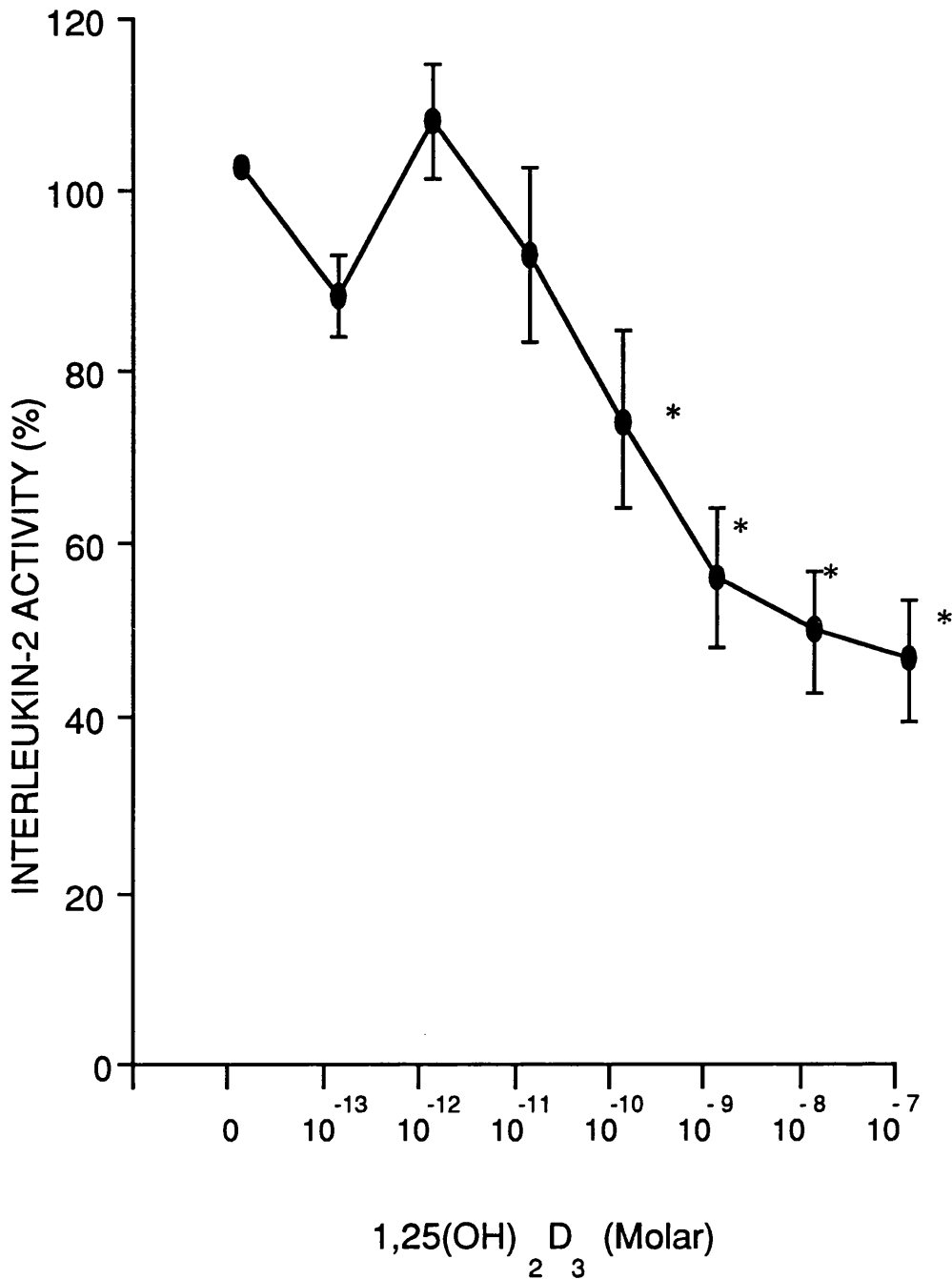


FIGURE 4.5.1.

The figure represents the IL-2 activity present in supernatants produced in the presence of 1,25 dihydroxyvitamin D₃. The IL-2 activity is presented as the percentage of control data i.e. when the supernatants were obtained in the presence of the carrier (ethanol). The data are the mean of 9 normal individuals. The standard error of the mean are represented as error bars. Statistics in the form of a paired Wilcoxon test was carried out (*p< 0.05).

Figure 4.5.1. depicts the inhibition of IL-2 by 1,25(OH)₂D₃. The IL-2 was expressed as a percentage of the control value (100 %). The inhibition of IL-2 activity occurs from 10⁻¹² M onwards and achieves significance at 10⁻¹⁰ M. The IL-2 continues to fall throughout the range. Local extra-renal synthesis of 1,25(OH)₂D₃ may result in concentrations in excess of normal sera (10⁻¹¹ M - 10⁻¹⁰ M) at particular sites in the body and thus the effect seen at higher concentrations could be of physiological importance.

4.6. THE EFFECTS OF 1,25(OH)₂D₃ AND SEX HORMONES ON INTERLEUKIN -2 ACTIVITY.

As demonstrated in section 3.5. the sex hormones can also inhibit lymphocyte activation in a manner similar to 1,25(OH)₂D₃. In section 4.5., 1,25(OH)₂D₃ was shown to inhibit IL-2 activity and this inhibition mirrored the inhibition of lymphocyte activation. A comparison of the effects of 1,25(OH)₂D₃ and the sex hormones, oestrogen, progesterone and dihydrotestosterone on IL-2 activity was undertaken.

4.6.1 IL-2 activity in the presence of 1,25(OH)₂D₃ and of various sex hormones.

PBMCs were obtained from four normal individuals by density gradient centrifugation (section 2.2.1.). The cells were cultured at a cell density of 10⁶ cells/ml in complete RPMI 1640 + 1 % F.C.S. in the presence of a range of 1,25(OH)₂D₃ concentrations or of a range of either of the sex hormones (oestrogen, progesterone or dihydrotestosterone). The cells were stimulated with 1 µg/ml PHA (Wellcome Grade) and cultured for 48 hours at 37 °C in a humidified atmosphere of 5 % CO₂/Air. After the incubation period the supernatants were harvested (section 2.8.1.) and stored at -20 °C. The amount of IL-2 in each of the supernatants was determined in the HT-2 bioassay (section 2.8.2.). The results were expressed both as units/ml and as percentage (%) of control activity.

Interleukin-2 Activity in the presence of 1,25 dihydroxyvitamin

D3, oestrogen, progesterone or dihydrotestosterone

Steroid Hormone Concentration (M)	1,25(OH) ₂ D ₃ IL-2 Activity (units/ml)	Oestrogen IL-2 Activity (units/ml)	Progesterone IL-2 Activity (units/ml)	Dihydrotestosterone IL-2 Activity (units/ml)
0	10.3 (3.6)	9.2 (3.1)	9.9 (3.7)	11.1 (4.7)
10 ⁻¹³	7.2 (2.5)	8.8 (3.1)	7.1 (2.7)	10.1 (4.8)
10 ⁻¹²	7.4 (2.7)	7.9 (2.9)	6.5 (2.4)	7.0 (3.2)
10 ⁻¹¹	5.1 (1.8)	5.9 (1.9)	5.9 (2.7)	6.0 (3.5)
10 ⁻¹⁰	4.9 (1.5)	7.3 (2.8)	7.1 (3.2)	7.2 (2.7)
10 ⁻⁹	4.3 (1.0)	7.9 (2.9)	7.2 (3.2)	6.3 (2.6)
10 ⁻⁸	3.7 (1.0)	8.8 (3.2)	7.6 (2.9)	8.0 (3.5)
10 ⁻⁷	3.8 (1.0)	11.0 (4.4)	10.9 (5.2)	10.8 (4.4)

TABLE 4.6.1.

The table presents the IL-2 activity present in supernatants produced in the presence of 1,25 dihydroxyvitamin D3 and the three sex hormones (oestrogen, progesterone and dihydrotestosterone). The IL-2 activity is presented as units/ml. The data are the mean of 4 normal individuals. The standard error of the mean are presented in parenthesis. Statistics were inappropriate.

Interleukin-2 activity expressed as a percentage of control in the presence of 1,25 dihydroxyvitamin D3, oestrogen, progesterone dihydrotestosterone

Steroid Hormone Concentration (M)	1,25(OH) ₂ D ₃ IL-2 Activity (% control)	Oestrogen IL-2 Activity (% control)	Progesterone IL-2 Activity (% control)	Dihydrotestosterone IL-2 Activity (% control)
0	100	100	100	100
10 ⁻¹³	72.4 (3.1)	95.4 (4.1)	71.3 (0.9)	85.0 (5.0)
10 ⁻¹²	69.8 (3.6)	82.1 (3.8)	65.7 (3.0)	59.9 (3.6)
10 ⁻¹¹	49.1 (4.5)	65.5 (2.0)	55.2 (4.8)	49.3 (8.6)
10 ⁻¹⁰	51.2 (4.4)	74.2 (5.8)	67.4 (6.7)	70.4 (4.6)
10 ⁻⁹	52.3 (10.1)	83.1 (4.0)	68.2 (11.3)	58.1 (5.6)
10 ⁻⁸	40.6 (5.0)	94.8 (5.7)	79.4 (7.2)	73.3 (5.0)
10 ⁻⁷	43.8 (6.6)	118.9 (14.7)	106.5 (14.6)	99.2 (2.1)

TABLE 4.6.2.

The figure presents the IL-2 activity present in supernatants produced in the presence of either 1,25 dihydroxyvitamin D3 or either of the sex hormones (oestrogen, progesterone or dihydrotestosterone). The IL-2 activities are presented as percentage of control. The data are the mean of 4 normal individuals. The standard error of the mean are represented as error bars. Statistics were inappropriate.

4.6.2. Sex hormones - A different pattern of inhibition.

The effects of 1,25(OH)₂D₃ and of the sex hormones on IL-2 activity are documented in table 4.6.1.. The first point is that the data follows much the same pattern as it did for lymphocyte activation. 1,25(OH)₂D₃ inhibits IL-2 activity throughout the range of concentrations of the metabolite tested. The sex hormones inhibit the IL-2 activity initially with the peak inhibition coming at 10⁻¹¹ M. The sex hormones then become less effective inhibitors of IL-2 activity, in a dose dependent manner. As a result at 10⁻⁷ M the sex hormones have no effect on the IL-2 activity the IL-2 activity has returned to control levels. In figure 4.6.1., the IL-2 activity is represented graphically and each of the sex hormones can be seen to match the 1,25(OH)₂D₃ over the range 0 M to 10⁻¹¹ M. From 10⁻¹⁰ M to 10⁻⁷ M the 1,25(OH)₂D₃ continues to inhibit the IL-2 activity whereas the sex hormones are less effective inhibitors. Table 4.6.2. presents the IL-2 activity results as percentage (%) control. This has the effect of minimising the error as the variation in the IL-2 activity between individuals was substantial when expressed as units/ml. The data was much tighter when presented in this form and confirms the conclusions derived from the units/ml data.

The effect of 1,25 dihydroxyvitamin D3 and the Sex hormones on IL-2 activity

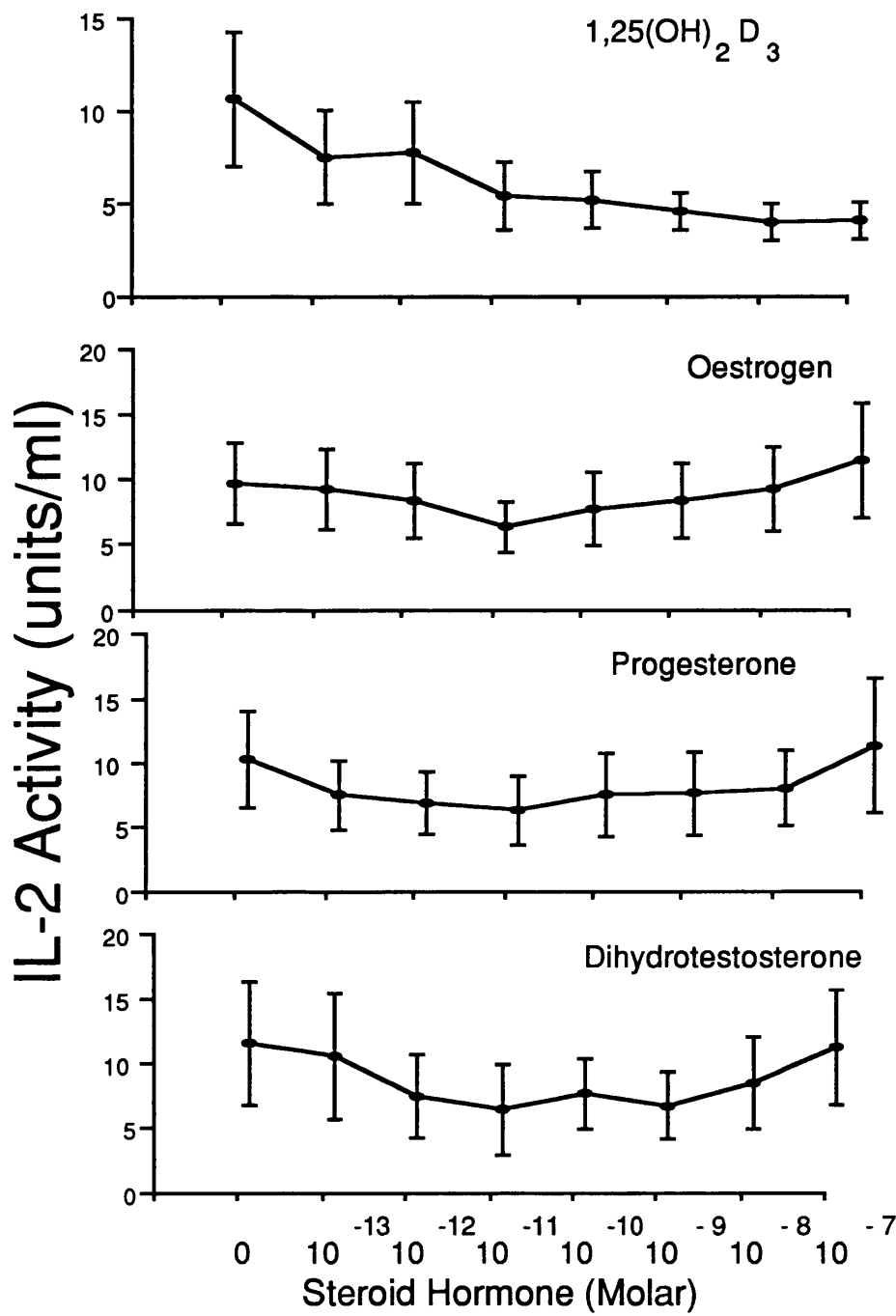


Figure 4.6.1.

The figure represents the IL-2 activity present in supernatants produced in the presence of either 1,25 dihydroxyvitamin D3 or either of the sex hormones (oestrogen, progesterone or dihydrotestosterone). The IL-2 activities are presented as units/ml. The data are the mean of 4 normal individuals. The standard error of the mean (SEM) are represented as error bars. Statistics were inappropriate.

The effect of 1,25 dihydroxyvitamin D3 and the Sex hormones on IL-2 activity expressed as a percentage

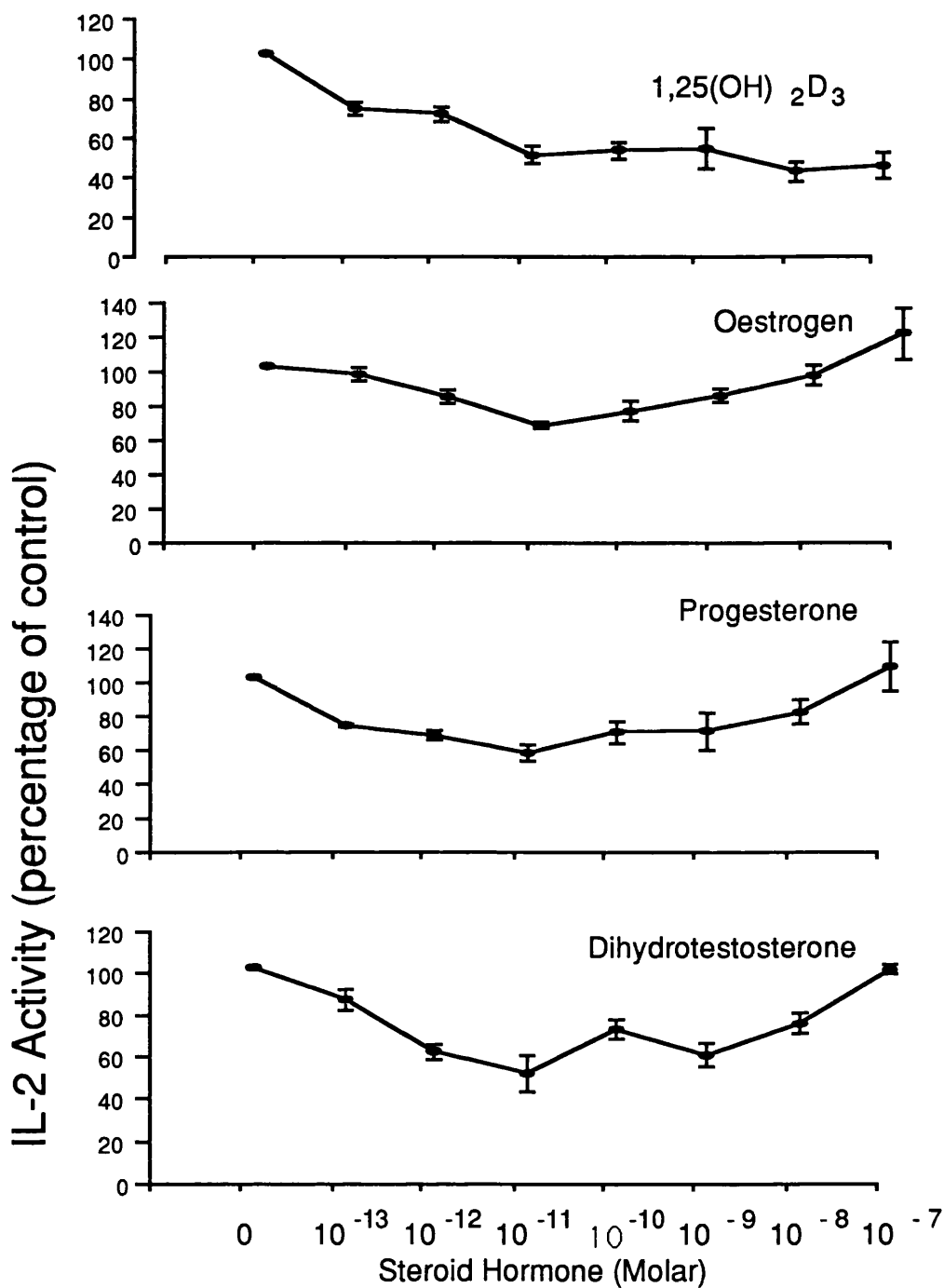


FIGURE 4.6.2.

The figure represents the IL-2 activity present in supernatants produced in the presence of either 1,25 dihydroxyvitamin D3 or either of the sex hormones (oestrogen, progesterone or dihydrotestosterone). The IL-2 activities are presented as percentage of control. The data are the mean of 4 normal individuals. The standard error of the mean (SEM) are represented as error bars. Statistics were inappropriate.

4.6.3. Inhibition by 1,25(OH)₂D₃ - A suggestion of a second mechanism.

1,25(OH)₂D₃ was a more potent inhibitor of IL-2 activity. All three sex hormones, however, match 1,25(OH)₂D₃ as regards inhibition over the range 10⁻¹³ M to 10⁻¹¹ M. At concentrations greater than 10⁻¹¹ M the sex hormones become less effective inhibitors of IL-2 activity. As was the case with the effects on lymphocyte activation the likely explanation was down regulation of the appropriate sex hormone receptor. It has to be remembered that like the sex hormones 1,25(OH)₂D₃ is a steroid hormone - a secosteroid. It is therefore possible that the inhibition observed over the range 10⁻¹³ M to 10⁻¹¹ M for each of the steroid hormones could be occurring through the same mechanism. Whereas with the sex hormones the down regulation of the steroid hormone receptor comes into play at 10⁻¹¹ M, this down regulation appears less important in the case of 1,25(OH)₂D₃. A second mechanism may be involved in the inhibition of both IL-2 activity and of lymphocyte activation (at concentrations in excess of 10⁻¹¹ M).

4.7. 1,25(OH)₂D₃ - DOES IT PREVENT SECRETION OF INTERLEUKIN - 2 ?

A plausible explanation for the role of 1,25(OH)₂D₃ in the inhibition of IL-2 activity is that it may be acting to prevent the secretion of IL-2 from the cell. As mentioned in the text earlier the mRNA for IL-2 produces a primary translational product that contains the classical hydrophobic signal sequence. This would suggest that the IL-2 is secreted in the same manner as other secretory proteins. It was therefore possible that 1,25(OH)₂D₃ is interfering with the post translational modification of the IL-2. This scenario was investigated, because the assay system was known to be sensitive to unmodified recombinant IL-2 it was safe to assume that the assay could recognise any IL-2 that is translated and awaiting post-translational modification.

4.7.1. Comparison of normal supernatants and supernatants from lysed cells.

PBMCs from seven normal individuals were obtained by density gradient centrifugation (section 2.2.1.). The cells were stimulated with 1 µg/ml PHA (Wellcome Grade) and cultured for 48 hours in the presence of a range of concentrations of 1,25(OH)₂D₃ (section 2.5.1.) at 37 °C in a humidified atmosphere of 5 % CO₂/Air. The cultures were set up in duplicate. One set of cells were harvested in the usual way (section 2.8.1.). The second set of cells were lysed by subjecting the cells to three cycles of freeze-thawing. The supernatant was then obtained by centrifugation followed by sterile filtering to remove cell debris. The supernatants from both harvesting methods were assessed for IL-2 activity using the HT-2 bioassay (section 2.8.2.).

4.7.2. Inhibition by 1,25(OH)₂D₃ still evident in supernatants from lysed cells.

Figure 4.7.1. documents the effect of 1,25(OH)₂D₃ on IL-2 activity in both the supernatants from whole cells and from lysed cells. 1,25(OH)₂D₃ inhibits IL-2 activity in both supernatant preparations. The curve obtained from the lysed cell supernatants was consistently lower than the curve obtained from the whole cell supernatants. There are two possible explanations for this ; one is that the IL-2 was sticking to the filter (which was possible considering the nature of the filters) the second is the possible release of inhibitor molecules during the lysis process that are binding to or competing with IL-2. The inhibitor molecules could take the form of the IL-2 receptor themselves. However, the fact remains that 1,25(OH)₂D₃ was still inhibiting IL-2 in the lysed supernatants implying that 1,25(OH)₂D₃ does not effect the secretion of IL-2. If 1,25(OH)₂D₃ was inhibiting the IL-2 secretion then it would have been expected that the IL-2 activity would be constant throughout the range of 1,25(OH)₂D₃. It is therefore unlikely that 1,25(OH)₂D₃ was involved in the post-translational modification of IL-2. Interpretation of these results is made difficult by the fact that the signal sequence may indeed render the molecule inactive in the assay system.

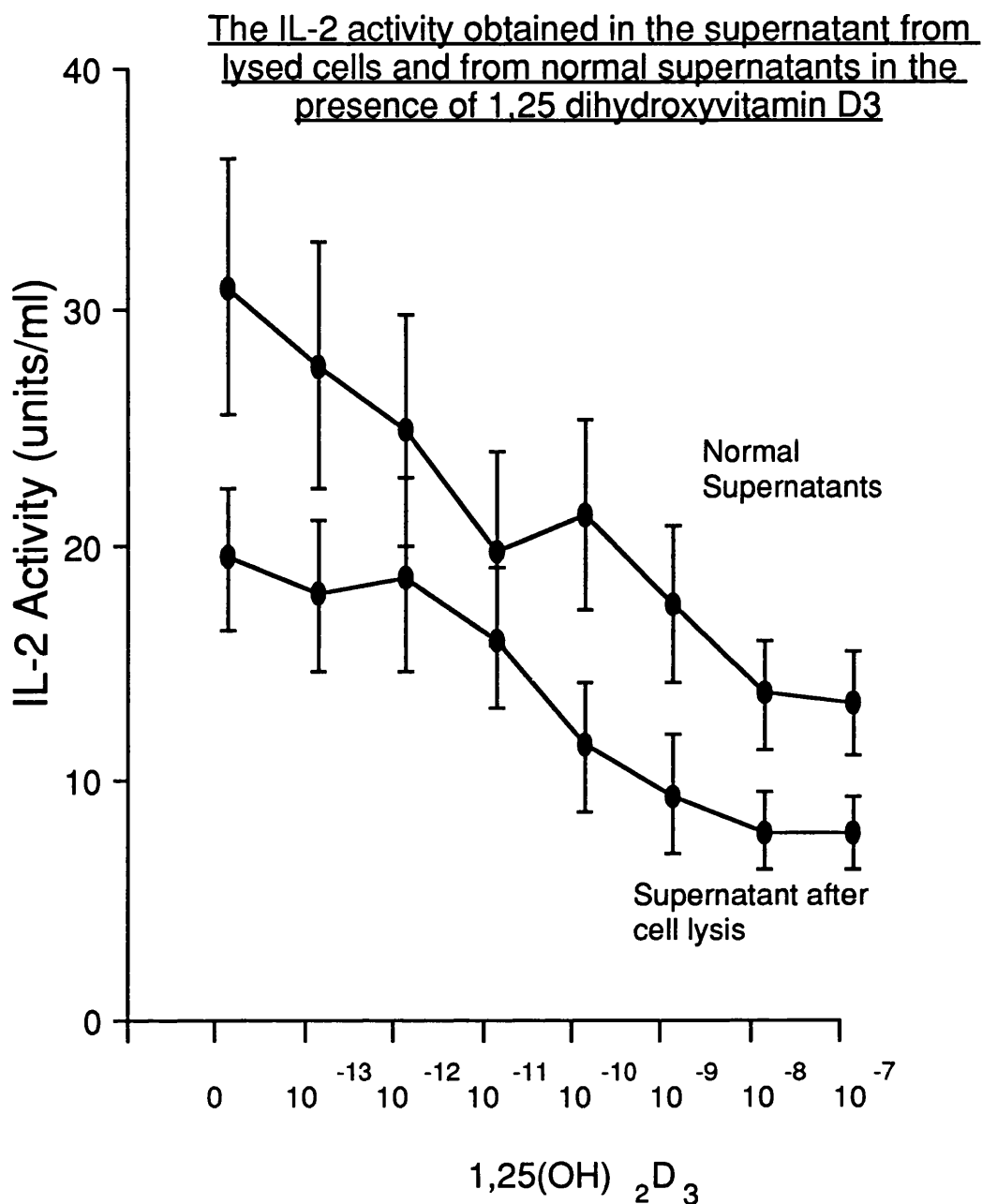


FIGURE 4.7.1.

The figure represents the IL-2 activity present in supernatants produced in the presence of 1,25 dihydroxyvitamin D₃. The supernatants were produced either by spinning down the cells or by subjecting the cells to three freeze - thaw cycles followed by centrifugation. The IL-2 activities are presented as units/ml. The data are the mean of 7 normal individuals. The standard error of the mean (SEM) are represented as error bars.

4.8. 1,25(OH)₂D₃ - DOES IT INTERFERE WITH THE ACTIVATION PROCESS ?

When the known facts are considered it is difficult to envisage 1,25(OH)₂D₃ mediating its effect through the signal induction pathway. It is known that resting lymphocytes do not possess the vitamin D receptor (VDR) nor for that matter the high affinity IL-2 receptor (IL-2R). Activated lymphocytes, on the other hand, do possess both receptors. The only conceivable way that 1,25(OH)₂D₃ could alter the lymphocyte activation process by antigen or mitogen is to prevent the mitogen delivering its signal to the membrane. This would imply that the 1,25(OH)₂D₃ has to function outside the cell.

4.8.1. PHA - Does it have to be present throughout ?

PBMCs from three normal individuals were obtained by density gradient centrifugation (section 2.2.1.). IL-2 containing supernatants were produced in two distinct ways. The first series of cells were set up in the usual way. The cells were resuspended at a cell density of 10⁶ cells/ml in complete RPMI 1640 and stimulated with 1 µg/ml PHA (Wellcome Grade). The cells were then cultured in the presence of a range of 1,25(OH)₂D₃ concentrations (section 2.5.1.) at 37 °C in a humidified atmosphere of 5 % CO₂/Air for 48 hours. The second series of cells were resuspended at a cell density of 10⁶ cells/ml in complete RPMI 1640 and stimulated with 1 µg/ml PHA (Wellcome Grade). The cells were incubated at 37 °C in a humidified atmosphere of 5 % CO₂/Air for four hours. The cells were washed three times in complete RPMI 1640 to remove as much of the PHA as was possible. These cells were then cultured in the presence of the range of 1,25(OH)₂D₃ concentrations for a further 44 hours at 37 °C in a humidified atmosphere of 5 % CO₂/Air. At the end of their respective incubations the IL-2 containing supernatants were harvested (section 2.8.1.). The IL-2 activity present was assessed in the HT-2 bioassay (section 2.8.2.).

4.8.2. Inhibition by 1,25(OH)₂D₃ - Not an effect on the activation signal.

The curves shown in figure 4.8.1. represent the effect of 1,25(OH)₂D₃ on IL-2 activity of cells cultured for 48 hours. One set of data refers to when the cells were stimulated with PHA for 48 hours and cultured in the presence of a range of 1,25(OH)₂D₃ concentrations throughout. The second set of data was obtained from cells stimulated with PHA for four hours and cultured for the remainder of the time in the presence of a range of 1,25(OH)₂D₃. Irrespective of whether the PHA was present on its own for four hours or was present throughout the incubation, 1,25(OH)₂D₃ still inhibited the IL-2 activity. The IL-2 activity in the supernatants derived from the cells stimulated with PHA for four hours prior to addition of 1,25(OH)₂D₃ was lower than the supernatants derived from cells stimulated with PHA throughout. A possible explanation is that the PHA stimulus could continually stimulate the cells for 48 hours hence the higher IL-2 activity in this set up. Figure 4.8.1. represents the IL-2 activity for one individual considered to be representative of the three individuals tested. Statistical tests are thus inappropriate.

The inhibition of IL-2 activity by 1,25(OH)₂D₃ can therefore not be attributed to an effect on the activation signal. If 1,25(OH)₂D₃ was affecting the activation signal then stimulation with PHA for four hours followed by a culture period in the presence of 1,25(OH)₂D₃ should not produce an inhibition of the IL-2 activity. Clearly there was still an inhibition, it was therefore reasonable to assume that 1,25(OH)₂D₃ was acting at a later stage possibly after the appearance of the VDR in the activated lymphocyte population.

The effect of 1,25 dihydroxyvitamin D3 on IL-2 activity when cells were stimulated either for the whole culture period or only for the initial four hours

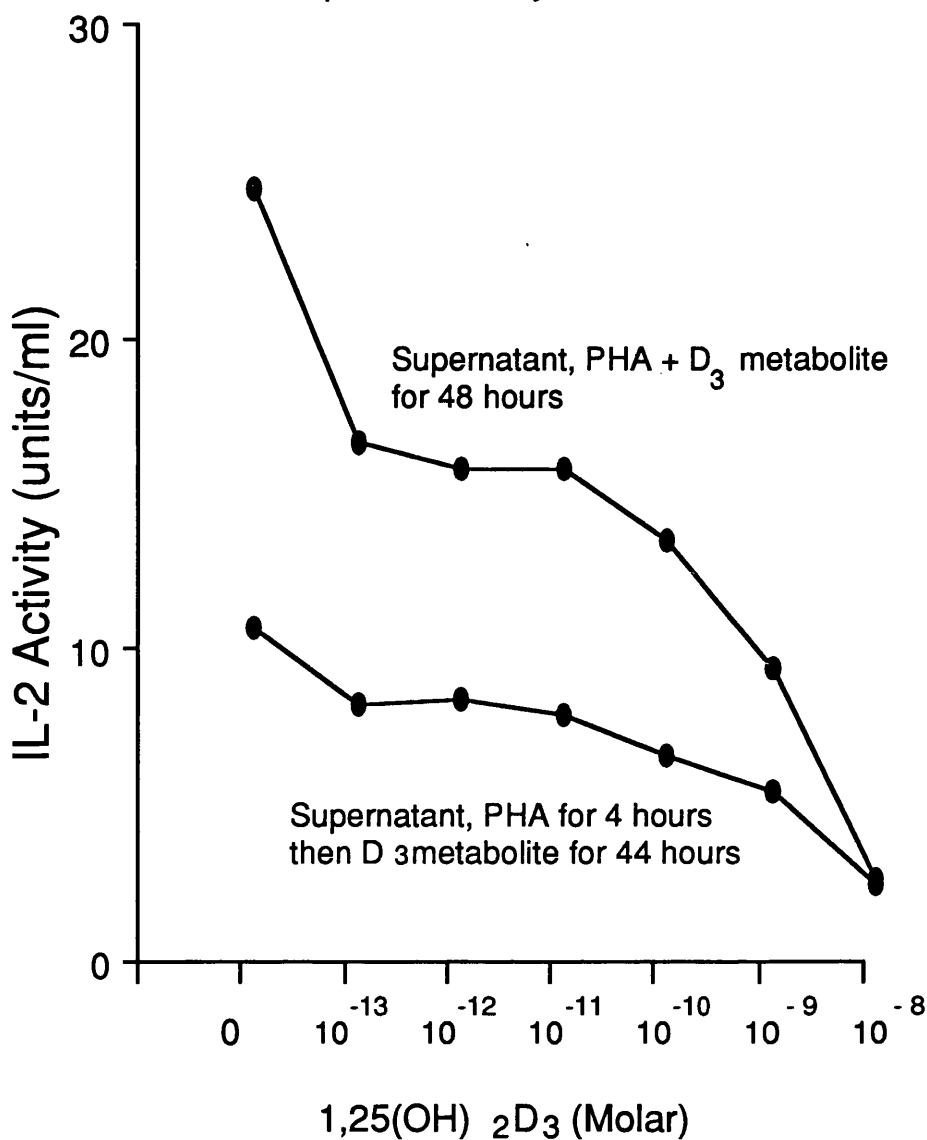


FIGURE 4.8.1.

The figure represents the IL-2 activity present in supernatants produced in the presence of 1,25 dihydroxyvitamin D₃. The supernatants were produced either by stimulating with PHA in the presence of 1,25(OH)₂D₃ for 48 hours or by stimulating with PHA alone for 4 hours, followed by culture in the absence of PHA but in the presence of 1,25(OH)₂D₃ for a further 44 hours. The IL-2 activities are presented as units/ml. The data are the results from 1 representative normal individual.

4.9. INTERLEUKIN -2 ACTIVITY - A GENUINE INHIBITION BY 1,25(OH)₂D₃.

Following on from chapter 3 where 1,25(OH)₂D₃ was established as the most effective calcitrophic hormone as regards lymphocyte transformation, it has been established that the same is true for IL-2 activity. PTH and CT failed significantly to alter IL-2 activity. 24,25(OH)₂D₃ and 25(OH)D₃ did achieve a significant inhibition of IL-2 activity, but this occurred at the higher concentrations tested (10⁻⁷ M). The inhibition of IL-2 activity correlates with the affinity for the D₃ metabolites for the vitamin D receptor (VDR). 1,25(OH)₂D₃ is therefore probably mediating its effect through the VDR.

The inhibition of IL-2 activity by 1,25(OH)₂D₃ occurs within the physiological range of this metabolite i.e. between 10⁻¹¹ M and 10⁻¹⁰ M in sera. Given that extra-renal synthesis of 1,25(OH)₂D₃ has been reported in monocytes it is possible that concentrations in excess of 10⁻¹⁰ M could be attained in the local microenvironment. It was clear that the inhibition of IL-2 activity by 1,25(OH)₂D₃ could be an important factor in immunoregulation. It was important to establish that the observed inhibition was not an artifact of the *in vitro* system, the fact that 1,25(OH)₂D₃ could inhibit IL-2 activity after lymphocyte activation was initiated suggests that the effect was not due to some interaction between the 1,25(OH)₂D₃ and the activation signal (PHA) and therefore the effect was genuine.

It has been suggested that 1,25(OH)₂D₃ may affect the secretion of the cytokine i.e. acts at the level of post-translational modification. This is unlikely as the supernatant produced from lysed cells exhibited an inhibition of the IL-2 activity. It is therefore possible to assume that the major effect of 1,25(OH)₂D₃ does not occur through effects on post-translational events in the production of IL-2.

CHAPTER 5.

1,25 DIHYDROXYVITAMIN D3 AND OTHER CYTOKINES.

5.1. OTHER CYTOKINES.

The inhibition of interleukin-2 (IL-2) by $1,25(\text{OH})_2\text{D}_3$ has been demonstrated in the previous chapter. It was now important to establish whether or not this effect was unique to IL-2 or whether other cytokines were influenced by $1,25(\text{OH})_2\text{D}_3$. The activation of lymphocytes in the immune system is intimately involved with cells of the monocyte/macrophage ($\text{M}\phi$) lineage. As a consequence the effects of $1,25(\text{OH})_2\text{D}_3$ on the monokines, interleukin-1 (IL-1) and interleukin-6 (IL-6) were studied.

5.1.1. Interleukin - 1 (IL-1).

Interleukin - 1 (IL-1) was initially described as a factor that acted as a co - mitogen for murine thymocytes stimulated with suboptimal concentrations of either PHA or Con A (Gery, 1972). Originally IL-1 was believed to be a product of activated macrophages (M ϕ) : now, it has become evident that IL-1 activities can be produced by virtually every nucleated cell type. IL-1 is produced by fibroblasts, endothelial cells, keratinocytes and smooth muscle to name but a few. The major source, however, remains the macrophage. It has been demonstrated that IL-1 is synthesised as a 33 kilodalton (kD) precursor that is processed to give a final product that ranges from 13 - 17 kD (Giri, 1985). There are two distinct species of IL-1, these are α and β . The α (271 amino acids) and the β (269 amino acids) exhibit limited sequence homology (22 - 26 %) (Mizel, 1988) although this sequence homology occurs at the biologically active carboxy-terminus. The α and β molecules have also different isoelectric points (pI for α = 5, pI for β = 7). The pathway for IL-1 secretion is poorly understood, but it is known that the precursor for IL-1 lacks the classical hydrophobic sequence (Lomedico, 1987). As a consequence of the absence of the 15 - 17 kD protein inside the cell it is assumed that the processing of the precursor occurs either at the membrane or after secretion.

The target cells for IL-1 are many and varied but all share a common involvement in immune or inflammatory responses. IL-1 has been reported to effect fibroblasts, synovial cells, chondrocytes, endothelial cells, hepatocytes and osteoclasts outside the immune system. However, the target cell that concerns most scientists is the T-cell. The CD4 +ve T_H cells respond to the dual signal of antigen in association with major histocompatibility complex (MHC) class II and IL-1 generated by the monocyte. The result of this sequence is to allow the T-cell to proliferate and to release a variety of cytokines including IL-2. It was for these reasons that the effects of 1,25(OH)₂D₃ on IL-1 activity in PBMCs were studied.

5.1.2. Interleukin-6 (IL-6).

Like IL-1, interleukin-6 (IL-6) has a broad range of target cells and can influence the immune system in many ways. Indeed, it has previously been called ; hybridoma growth factor (HGF), plasmacytoma growth factor, hepatocyte stimulating factor, B-cell stimulating factor 2 and $\beta 2$ -interferon (Billiau, 1989; Kishimoto, 1988). Also like IL-1, IL-6 is a glycoprotein that is heterogeneous in size (23 - 30 kD). This heterogeneity is a consequence of differential glycosylation. The protein has a structure of 212 amino acids, the first 28 form the classic hydrophobic signal sequence. IL-6 is produced by a variety of cells, which include monocyte/macrophages, fibroblasts, endothelial cells, T and B cells, mesangial cells, keratinocytes and a number of tumour types. Normal cells do not produce IL-6 unless the cells are stimulated. IL-6 production can be stimulated by a variety of factors including IL-1, tumour necrosis factor α (TNF α), platelet derived growth factor (PDGF), virus infection, double stranded RNA and cyclic AMP.

Generally, IL-6 is produced during the immune response and can effect both T-cell and B-cell differentiation. The effects of IL-6 are mostly seen when there is another stimulus present - more often than not, IL-1. Lymphocyte activation, as it happens, provides an excellent example of a synergistic effect between cytokines. The stimulation of lymphocyte proliferation by IL-6 involves a direct growth promoting signal and also an induction of IL-2 receptors. IL-6 is also involved with cytotoxic T-cells - the cytokine induces the generation of these cell types. This agrees with the postulated role for IL-6 in antiviral activity. IL-6 has also been implicated in B-cell growth and differentiation into antibody secreting cells. As a consequence of the key role that IL-6 has on lymphocyte activation, the effects of $1,25(\text{OH})_2\text{D}_3$ were studied in the production of this cytokine.

5.2. INTERLEUKIN - 1 ACTIVITY - DOES 1,25(OH)₂D₃ HAVE AN EFFECT ON INTERLEUKIN - 1 ACTIVITY.

Most of the work up to this point was carried out on PBMC's and therefore the cell population was mixed. It was important to establish whether or not 1,25(OH)₂D₃ was acting on monocytes and the inhibition was mediated through these cells. PHA was used in order that the systems could be compared.

5.2.1. The effect of 1,25(OH)₂D₃ on IL-1 activity.

PBMCs were obtained from eight normal individuals by density gradient centrifugation (section 2.2.1.). The cells were resuspended at 10⁶ cells/ml in complete RPMI 1640 + 2 % F.C.S. and were either stimulated with PHA (1 µg/ml) or were unstimulated. The cells were cultured in the presence of a range of concentrations for 1,25(OH)₂D₃ (10⁻¹³ M - 10⁻⁷ M). The cells were cultured overnight (approximately 18 hours) at 37 °C in a humidified atmosphere of 5 % CO₂/Air. The supernatants were harvested and stored at -20 °C until assayed for IL-1. An overnight incubation was chosen to maximise IL-1 while minimising IL-2 activity.

The IL-1 present in the supernatants was assessed using the IL-1 bioassay (section 2.9.3.). The samples were set up in triplicate as four dilutions of the supernatant (final dilutions in the assay of 1 in 10, 1 in 100, 1 in 1000 and 1 in 10000). The standard curve was constructed from an in - house standard (section 2.9.2.) that had been calibrated against the standard obtained from the National institute of Biological Standards. The standard curve was made up of ten two - fold dilutions of a 1 in 100 dilution of the standard preparation (section 2.9.2.).

5.2.2. IL-1 activity - Very little change.

The IL-1 activity in the presence of various concentrations of 1,25(OH)₂D₃ in both stimulated cells (PHA) and unstimulated cells is presented in table 5.2.1.. There was not much difference in the amount of IL-1 activity produced by either stimulated or unstimulated cells throughout the range of 1,25(OH)₂D₃ concentrations. The IL-1 activity in unstimulated cells was significantly inhibited at 10⁻¹⁰ M 1,25(OH)₂D₃ but the IL-1 activity returned to control levels at higher concentrations of 1,25(OH)₂D₃. The stimulated cells, however, were significantly inhibited at both 10⁻¹³ M and 10⁻¹¹ M 1,25(OH)₂D₃. At higher concentrations of 1,25(OH)₂D₃ the IL-1 activity returned to control levels. The results are also represented in figure 5.2.1.. The inhibition of IL-1 activity by 1,25(OH)₂D₃ observed in both stimulated and unstimulated cells appears to be minor compared to the inhibition of IL-2 and lymphocyte activation by 1,25(OH)₂D₃ (section 4.5. and section 3.4.).

5.2.3. Minimal contribution by IL-1 to the inhibition of IL-2 by 1,25(OH)₂D₃.

Although at some concentrations of 1,25(OH)₂D₃ there appears to be a minor inhibition of IL-1 activity, there does not appear to be enough of an effect to account for the inhibition of IL-2 by 1,25(OH)₂D₃. The observation that there was very little difference between the stimulated cells and the unstimulated cells argues that the contribution made by PHA stimulation on IL-1 activity was negligible and consequently strengthens the argument for the inhibition of IL-2 by 1,25(OH)₂D₃ being a real effect in the system.

Interleukin-1 activity produced in cells cultured in the presence of 1,25 dihydroxyvitamin D3 and either stimulated or unstimulated with PHA

1,25(OH) ₂ D ₃ (Molar)	IL-1 activity (units/ml) Unstimulated	IL-1 activity (units/ml) Stimulated
0	256.8 (92.8)	309.3 (100.2)
10 ⁻¹³	194.8 (69.1)	213.5* (62.5)
10 ⁻¹²	201.1 (60.7)	234.7 (79.1)
10 ⁻¹¹	149.3 (46.8)	206.5 (63.9)
10 ⁻¹⁰	193.5* (70.3)	224.8 (68.7)
10 ⁻⁹	250.0 (108.1)	259.1 (78.3)
10 ⁻⁸	263.3 (97.4)	332.1 (125.3)
10 ⁻⁷	250.4 (90.5)	238.9 (67.9)

Table 5.2.1.

The table presents the IL-1 activity present in supernatants produced in the presence of 1,25 dihydroxyvitamin D3 when stimulated with PHA or unstimulated. The data were obtained from 8 normal individuals and are presented as the mean with the standard error of the mean in parenthesis. The IL-1 activity is measured in units/ml. Statistics took the form of a paired Wilcoxon test (* p < 0.05) and compared 0 with all other concentrations of 1,25(OH)₂D₃.

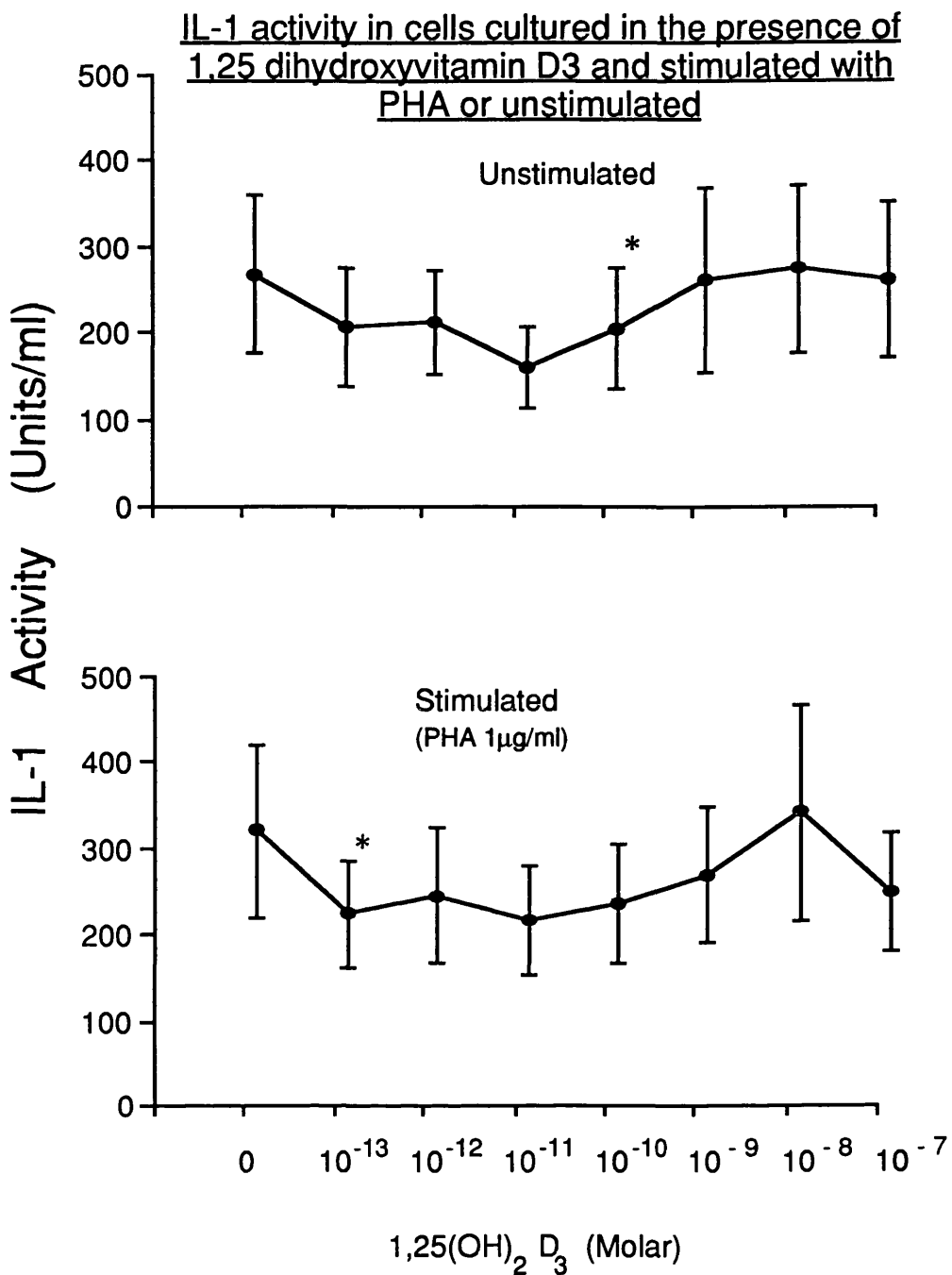


FIGURE 5.2.1.

The figure represents the IL-1 activity in units/ml of cells that were either stimulated with PHA or unstimulated and cultured in the presence of 1,25 dihydroxyvitamin D3. The data were obtained from 8 normal individuals. The standard error of the mean (SEM) are represented as error bars. The paired Wilcoxon statistical test was used to ascertain the significance of the results (*p> 0.05) comparing 0 with all other concentrations of 1,25 dihydroxyvitamin D3.

5.3. LIPOPOLYSACCHARIDE - A MORE APPROPRIATE STIMULUS FOR INTERLEUKIN - 1 ACTIVITY.

Monocytes are normally stimulated with lipopolysaccharide (LPS) which substitutes for the bacterial cell wall that acts as a stimulus *in vivo*. In this section the effects of 1,25(OH)₂D₃ on IL-1 activity produced by PBMCs stimulated with LPS were studied.

5.3.1. The effect of 1,25(OH)₂D₃ on LPS induced IL-1 activity.

PBMCs were obtained from seven normal individuals by density gradient centrifugation (section 2.2.1.). The cells were resuspended at 10⁶ cells/ml in complete RPMI 1640 + 5 % F.C.S. and stimulated with 10 µg/ml LPS. The cells were cultured in the presence of a range of concentrations of 1,25(OH)₂D₃ (10⁻¹³ M - 10⁻⁷ M) at 37 °C for 18 hours in a humidified atmosphere of 5 % CO₂/Air. The supernatants were harvested and stored at -20 °C until assayed.

The IL-1 activity was assessed in the LBRM TG6/HT-2 bioassay (section 2.9.3.). The samples were set up in duplicate as eight dilutions five - fold dilutions (1 in 5, 1 in 25, 1 in 125, 1 in 625, 1 in 3125, 1 in 15625, 1 in 78125 and 1 in 390625). The standard curve was constructed from a two - fold serial dilution of a 1 in 100 dilution of the in - house IL-1 standard preparation (section 2.9.2.).

5.3.2. 1,25(OH)₂D₃ - No real change in IL-1 activity.

The IL-1 activity in supernatants derived from PBMCs stimulated with 10 µg/ml LPS and cultured in the presence of a range of 1,25(OH)₂D₃ concentrations is presented in table 5.3.1.. IL-1 activity was initially enhanced by 1,25(OH)₂D₃ with the peak enhancement occurring at 10⁻¹² M with the IL-1 activity returning to control levels at higher concentrations of 1,25(OH)₂D₃ (10⁻⁸ M). At 10⁻⁷ M the IL-1 activity appeared to be inhibited. The data is also presented in figure 5.3.1..

5.3.3. Inhibition of IL-2 by 1,25(OH)₂D₃ - No IL-1 involvement.

These observations were the opposite of the ones presented in the previous section. The main difference between the two experiments are the method of stimulation. The important observation was that the effect of 1,25(OH)₂D₃ on IL-1 activity is not a dose dependent effect in either case. This argues that the effect that was seen with 1,25(OH)₂D₃ on IL-2 activity is not mediated through IL-1 activity.

Interleukin-1 activity produced by cells cultured in
the presence of 1,25 dihydroxyvitamin D3 and stimulated
with LPS

1,25(OH) ₂ D ₃ (Molar)	IL-1 activity (units/ml)
0	897.3 (188.7)
10 ⁻¹³	827.1 (233.1)
10 ⁻¹²	1464.6 (713.9)
10 ⁻¹¹	1193.7 (428.4)
10 ⁻¹⁰	1022.1 (256.8)
10 ⁻⁹	1001.7 (169.1)
10 ⁻⁸	916.8 (224.9)
10 ⁻⁷	585.7 (133.9)

TABLE 5.3.1.

The table presents the IL-1 activity present in supernatants produced in the presence of 1,25 dihydroxyvitamin D3 when stimulated with LPS. The data were obtained from 7 normal individuals and are presented as the mean with the standard error of the mean in parenthesis. The IL-1 activity is measured in units/ml. Statistics took the form of a paired Wilcoxon test (* p < 0.05) and compared 0 with the other concentrations of 1,25(OH)₂D₃.

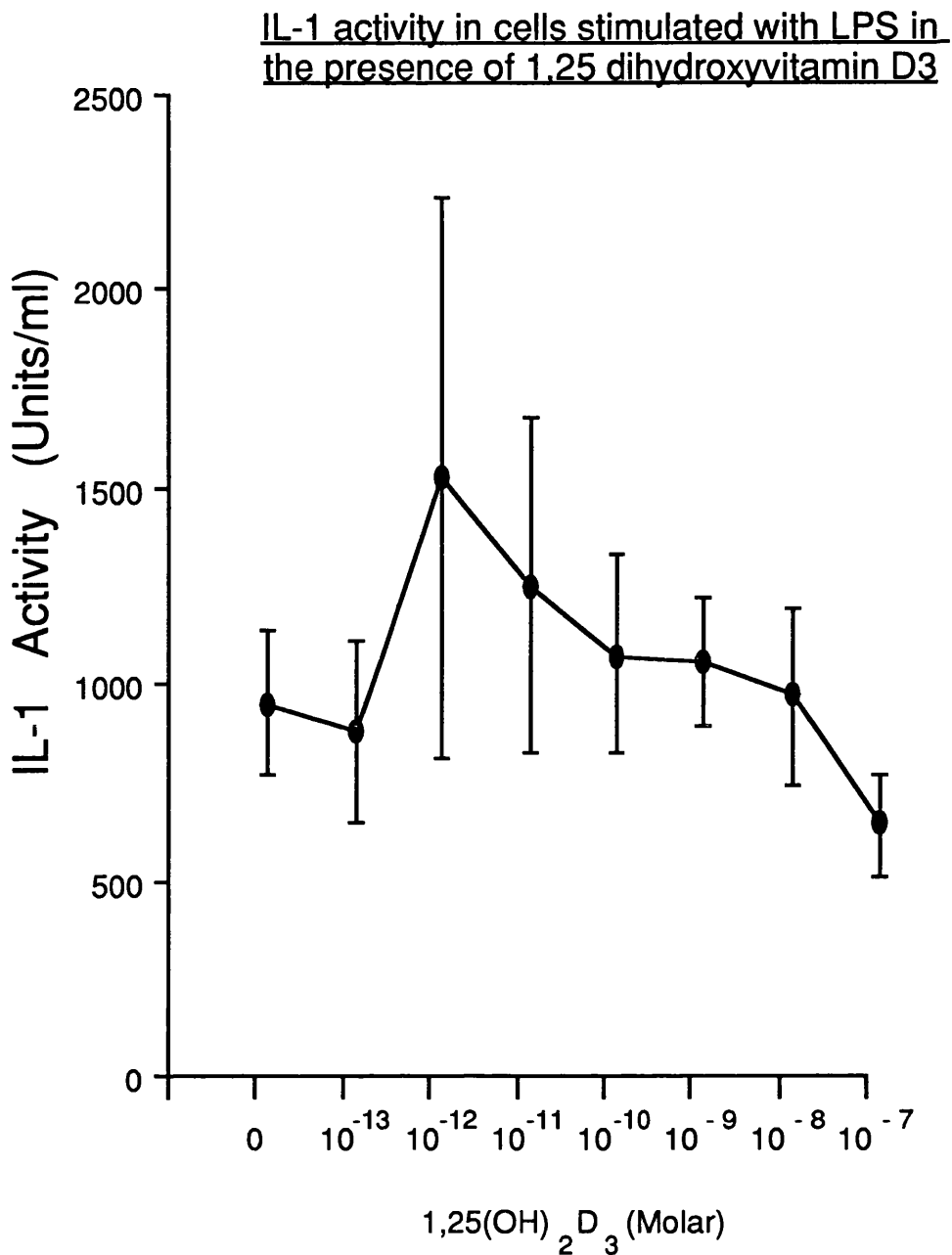


FIGURE 5.3.1.

The figure represents the IL-1 activity present in supernatants produced in the presence of 1,25 dihydroxyvitamin D3 when stimulated with LPS. The data were obtained from 7 normal individuals and are presented as the mean with the standard error of the mean (SEM) represented as error bars. The IL-1 activity is measured in units/ml. Statistics in the form of a paired Wilcoxon test comparing 0 to all other concentrations of 1,25 dihydroxyvitamin D3 revealed no significant differences.

5.4. NO EFFECT ON INTERLEUKIN - 1 ACTIVITY - A GENUINE OBSERVATION.

The pattern of results obtained in section 5.3. was further investigated to ascertain whether the effect is genuine or an artifact arising from the stimulation with LPS (PHA as a stimulus does not effect HT-2 cells). It could be that the LPS present in the supernatant interferes with the LBRM TG6/HT-2 bioassay. To investigate this possibility the supernatants produced in section 5.3 were also assayed on the IL-1 β ELISA kit obtained from Cistron Biotechnology, New Jersey, U.S.A.. This assay being an ELISA should not be influenced by excess LPS present in the supernatants.

5.4.1. IL-1 activity as measured by an ELISA.

The assay was set up as described in section 2.9. and the samples were diluted 1 in 10 in complete RPMI 1640. The standards and the diluted samples were carried out in duplicate.

5.4.2. Assays produce same trend.

Table 5.4.1. depicts the IL-1 β activity present in the supernatants obtained in section 5.3.. Clearly the pattern is the same so the results obtained in section 5.3. would appear to be genuine and the difference between the IL-1 activity in cells stimulated with PHA and LPS is a consequence of the stimulation signal. Figure 5.4.1. demonstrates that the graph obtained is essentially the same as figure 5.3.1..

Interleukin-1 activity produced by cells cultured in
the presence of 1,25 dihydroxyvitamin D3 and stimulated
with LPS assessed by an ELISA method

1,25(OH) ₂ D ₃ (Molar)	IL-1 activity (units/ml)
0	108.3 (17.5)
10 ⁻¹³	123.1 (28.3)
10 ⁻¹²	151.8 (24.1)
10 ⁻¹¹	179.4 (31.9)
10 ⁻¹⁰	129.2 (34.3)
10 ⁻⁹	62.5 (18.8)
10 ⁻⁸	84.7 (17.5)
10 ⁻⁷	102.7 (16.1)

TABLE 5.4.1.

The table presents the IL-1 β activity present in supernatants produced in the presence of 1,25 dihydroxyvitamin D3 when stimulated with LPS. The data were obtained from 7 normal individuals and are presented as the mean with the standard error of the mean in parenthesis. The IL-1 β activity is measured in units/ml. Statistics took the form of a paired Wilcoxon test and compared 0 to all other concentrations of 1,25(OH)₂D₃. There was no significant differences throughout the range.

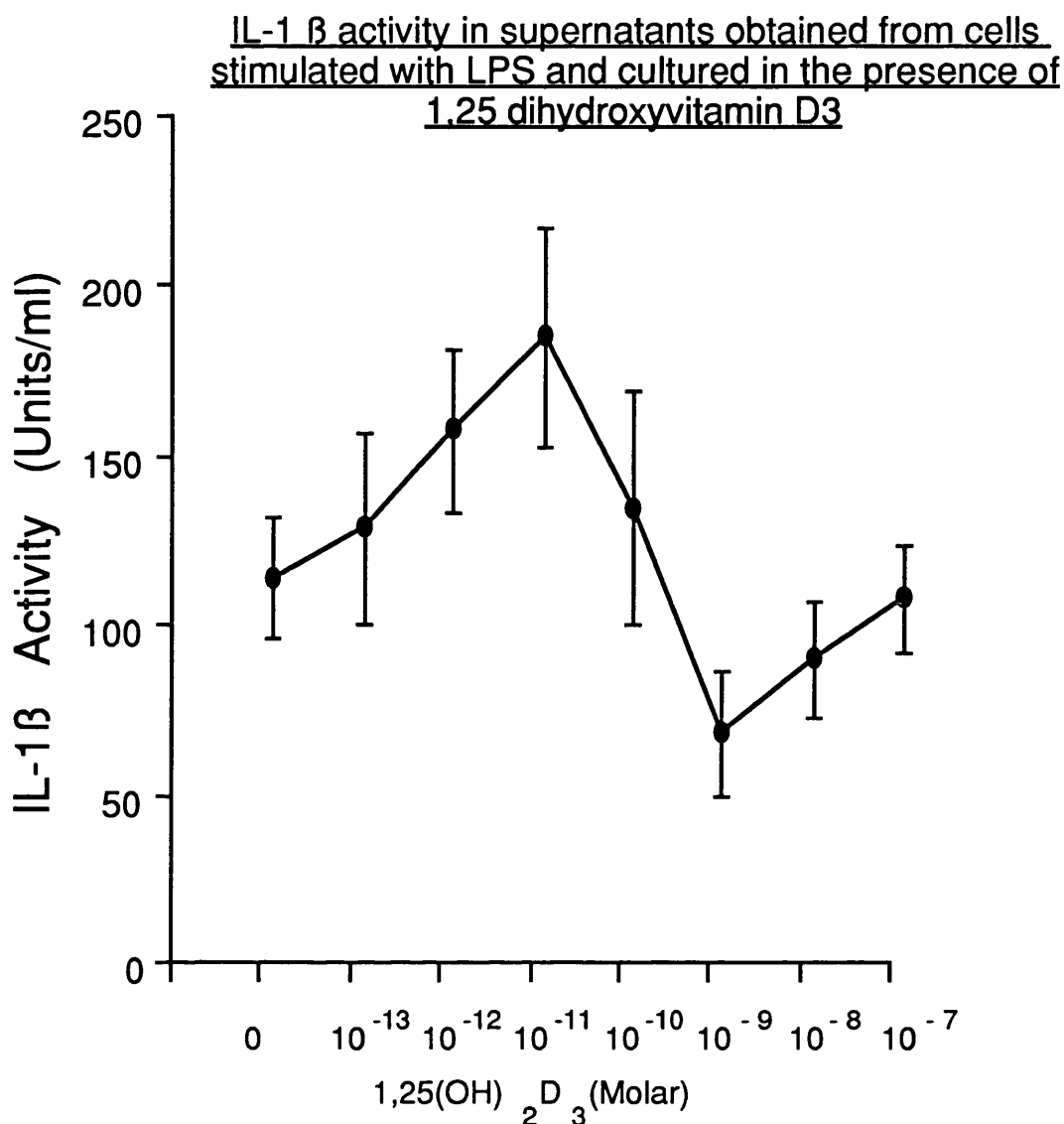


FIGURE 5.4.1.

The figure represents the IL-1 β activity present in supernatants produced in cells stimulated with 10 μ g/ml LPS and cultured in the presence of 1,25 dihydroxyvitamin D3. The data were obtained from 7 normal individuals and are presented as the mean with the standard error of the mean (SEM) represented as error bars. The IL-1 activity is measured in units/ml. Statistics in the form of a paired Wilcoxon test comparing 0 to all other concentrations of 1,25 dihydroxyvitamin D3 revealed no significant differences.

5.4.3. IL-1 activity can not account for the inhibition of IL-2 by 1,25(OH)₂D₃.

The stimulus for the production of IL-1 was important. Depending on the stimulus an increase or a decrease in IL-1 activity could be attained in the presence of 1,25(OH)₂D₃. The increase or decrease was not dose dependent and returned to control values at the higher concentrations of the 1,25(OH)₂D₃. The dose dependent effects of 1,25(OH)₂D₃ on both IL-2 activity and cell proliferation can not therefore be accounted for by changes in the IL-1 activity.

5.5. INTERLEUKIN - 6 ACTIVITY - DOES 1,25(OH)₂D₃ HAVE AN EFFECT ?

If IL-1 activity was not altered by 1,25(OH)₂D₃ then perhaps IL-6 activity could be. Like IL-1, IL-6 is produced by the monocyte and therefore could also be a candidate for a possible mediator of the inhibition of IL-2 by 1,25(OH)₂D₃.

5.5.1. The effect of 1,25(OH)₂D₃ on IL-6 activity.

PBMCs were obtained from eight normal individuals by density gradient centrifugation (section 2.2.1.). The cells were resuspended in complete RPMI 1640 at a cell density of 10⁶ cells/ml. The cells were either stimulated with 1 µg/ml PHA (Wellcome Grade) or were unstimulated. The cells were cultured in the presence of a range of concentrations of 1,25(OH)₂D₃ (10⁻¹³ M - 10⁻⁷ M). The cells were cultured for 18 hours at 37 °C in a humidified atmosphere of 5 % CO₂/Air. The supernatants were harvested and stored at -20 °C until required in the assay.

The IL-6 activity was assessed in the B9 bioassay (section 2.10.2.). The IL-6 containing supernatants were two - fold serially diluted in complete RPMI 1640. The B9 cells were incubated with these dilutions for 68 hours at which time MTT was added to each well (section 2.10.4.). The assay was assessed at 72 hours using the Dynatech MR 700 microplate reader.

5.5.2. No sustained effect on IL-6 activity.

Table 5.5.1. illustrates the IL-6 activity present in cells stimulated with PHA (1 µg/ml) and unstimulated cells cultured in the presence of a range of 1,25(OH)₂D₃ concentrations. There was very little difference in the IL-6 activity in cells that were stimulated as compared to their unstimulated counterparts. The stimulated cells were slightly higher than the unstimulated cells. Like the IL-1 activity the IL-6 activity appears to increase initially as the 1,25(OH)₂D₃ concentration is increased, also after 10⁻¹¹ M the activity falls back to control levels. Figure 5.5.1. represents the data graphically. Again the similarities between the IL-1 activities (figure 5.2.1.) and the IL-6 activities are quite striking. The effect of 1,25(OH)₂D₃ on both IL-2 activity and cell proliferation (section 4.5. and section 3.4.) can not be explained by an effect on IL-6 activity.

Interleukin-6 activity produced in cells cultured in the presence of 1,25 dihydroxyvitamin D3 and either stimulated or unstimulated with PHA

1,25(OH) ₂ D ₃ (Molar)	IL-6 activity (units/ml) Unstimulated	IL-6 activity (units/ml) Stimulated
0	431.4 (93.7)	481.2 (69.9)
10 ⁻¹³	481.8 (105.1)	515.6 (88.9)
10 ⁻¹²	526.8 (131.5)	552.4 (109.4)
10 ⁻¹¹	434.2 (90.0)	561.7 (145.9)
10 ⁻¹⁰	391.1 (68.0)	370.7 (86.3)
10 ⁻⁹	474.5 (85.7)	502.9 (141.4)
10 ⁻⁸	480.8 (94.5)	467.4 (106.3)
10 ⁻⁷	406.6 (63.6)	427.6 (106.2)

TABLE 5.5.1.

The table presents the IL-6 activity in cells either stimulated with PHA or unstimulated and cultured in the presence of 1,25 dihydroxyvitamin D3. The data are obtained from 7 normal individuals and are presented as the mean and the standard error of the mean are in parenthesis. The IL-6 activity was measured in units/ml.

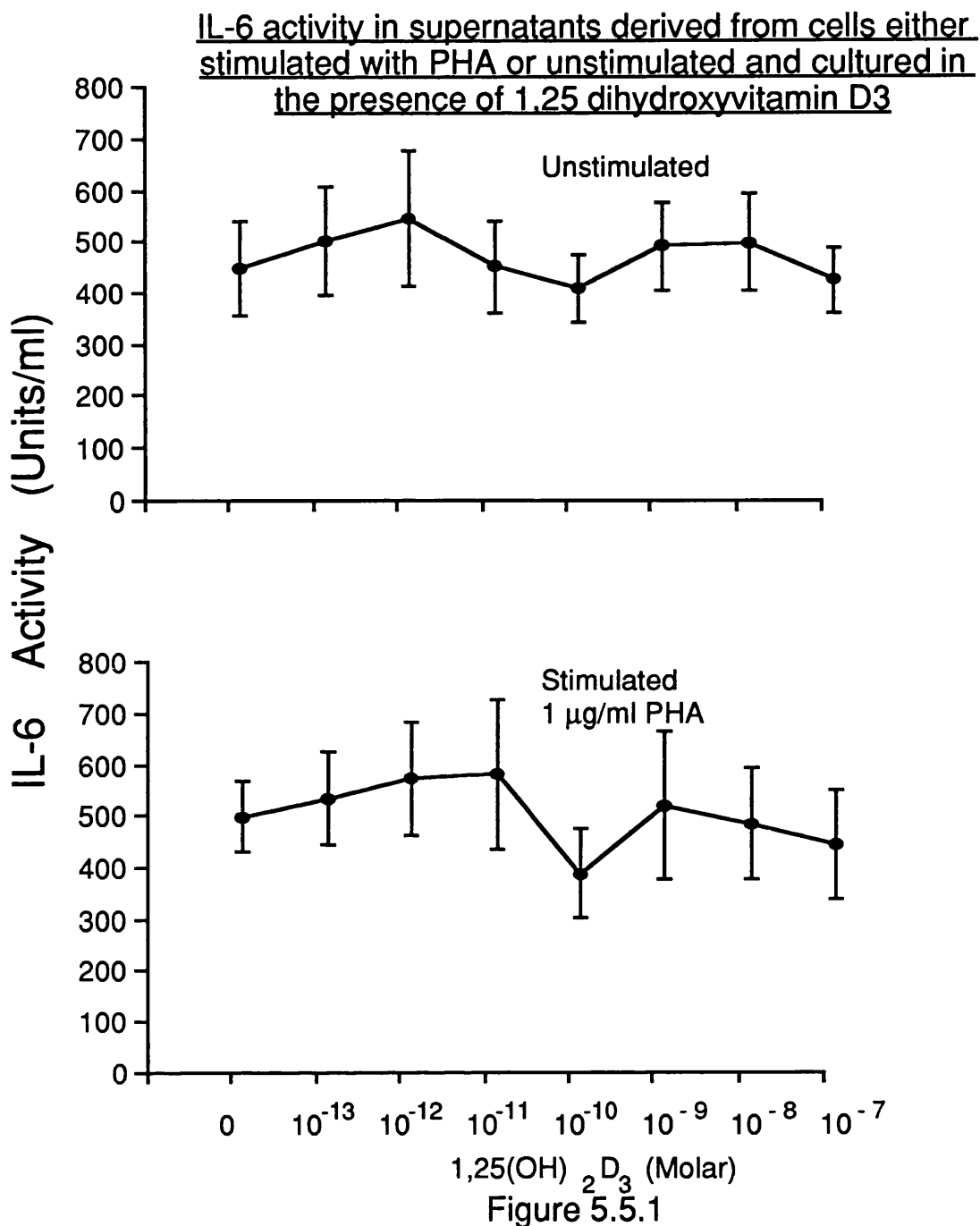


Figure 5.5.1

The figure represents the IL-6 activity present in supernatants derived by either from cells stimulated with PHA or unstimulated and cultured in the presence of 1,25 dihydroxyvitamin D₃. The data are obtained from 7 normal individuals and are presented as the mean with the standard error of the mean (SEM) portrayed as error bars. The IL-6 activity is measured in units/ml. Statistics in the form of a paired Wilcoxon test comparing 0 to all other concentrations of 1,25 dihydroxyvitamin D₃ revealed no significant differences.

5.6. LIPOPOLYSACCHARIDE - A MORE APPROPRIATE STIMULUS ?

The IL-6 activity in the presence of 1,25(OH)₂D₃ presented in section 5.5. was obtained using PBMCs stimulated with PHA. PHA is not a recognised stimulator of IL-6 so an experiment was set up to look at IL-6 activity in cells cultured in the presence of 1,25(OH)₂D₃ and stimulated with LPS.

5.6.1. The effect of 1,25(OH)₂D₃ on LPS induced IL-6 activity.

PBMCs were obtained from eight normal individuals by density gradient centrifugation (section 2.2.1.). The cells were resuspended in complete RPMI 1640 + 10 % F.C.S. at a cell density of 10⁶ cells/ml. The cells were stimulated with 10 µg/ml LPS and cultured in the presence of a range of concentrations of 1,25(OH)₂D₃ (10⁻¹³ M - 10⁻⁷ M). The cells were incubated at 37 °C in a humidified atmosphere of 5 % CO₂/Air for 18 hours. The supernatants were harvested and stored at -20 °C until required in the assay.

The supernatants were assayed in the B9 bioassay. The samples were two-fold serially diluted in complete RPMI 1640. The assay was incubated at 37 °C in 5 % CO₂/Air for a total of 72 hours. Four hours prior to the end of the incubation MTT was added to each well (section 2.10.4).

5.6.2. IL-6 activity not altered by 1,25(OH)₂D₃.

Table 5.6.1. lists the IL-6 activity of cells cultured in the presence of 1,25(OH)₂D₃ and stimulated with LPS. The IL-6 activity initially increases with increasing 1,25(OH)₂D₃ concentration with maximum production occurring at 10⁻¹¹ M. As in section 5.4. at the higher concentrations of 1,25(OH)₂D₃ the IL-6 activity returns to normal control levels. The data are presented in graph form in figure 5.6.1. and this resembles the figure 5.4.1.. The IL-6 activity in fact dips below the normal control levels at 10⁻⁸ M and 10⁻⁷ M.

Interleukin-6 activity produced by cells cultured in
the presence of 1,25 dihydroxyvitamin D3 and stimulated
with LPS

1,25(OH) ₂ D ₃ (Molar)	IL-6 activity (units/ml) (x 10 ⁻³)
0	90.7 (15.3)
10 ⁻¹³	106.7 (20.0)
10 ⁻¹²	105.5 (20.5)
10 ⁻¹¹	120.0 (16.2)
10 ⁻¹⁰	107.3 (11.0)
10 ⁻⁹	101.7 (18.8)
10 ⁻⁸	67.5 (9.7)
10 ⁻⁷	63.8 (8.4)

TABLE 5.6.1.

The table presents the IL-6 activity in supernatants from cells stimulated with LPS and cultured in the presence of 1,25 dihydroxyvitamin D3. The data are obtained from 8 normal individuals and are presented as the mean and the standard error of the mean are in parenthesis. The IL-6 activity was measured in units/ml.

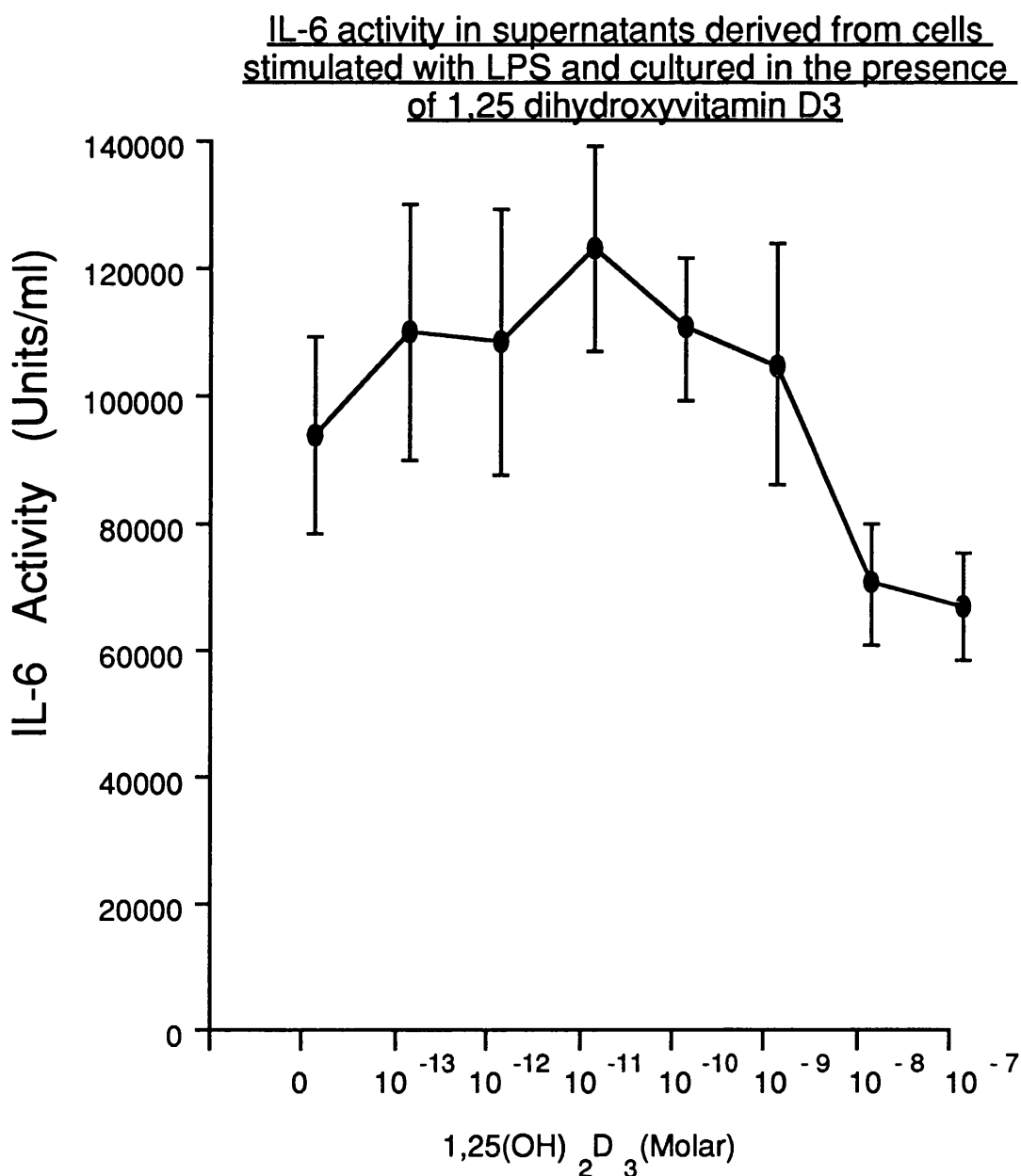


FIGURE 5.6.1.

The figure represents the IL-6 activity present in supernatants derived from cells stimulated with LPS and cultured in the presence of 1,25 dihydroxyvitamin D3. The data are obtained from 8 normal individuals and are presented as the mean with the standard error of the mean (SEM) portrayed as error bars. The IL-6 activity is measured in units/ml. Statistics in the form of a paired Wilcoxon test comparing 0 with all other concentrations of 1,25 dihydroxyvitamin D3 revealed no significant differences.

5.7. 1,25 DIHYDROXYVITAMIN D₃ ACTS DIRECTLY ON T - CELLS TO INHIBIT INTERLEUKIN - 2.

The effects of 1,25(OH)₂D₃ on both IL-1 and IL-6 are less clear cut than the effect of this metabolite of vitamin D on IL-2 activity. The IL-1 and IL-6 activities followed the same pattern when cultured in the presence of 1,25(OH)₂D₃. Initially at the lower concentrations of 1,25(OH)₂D₃ (10⁻¹³ M - 10⁻¹¹ M) both activities appeared to be enhanced. At the higher concentrations both activities returned to the normal control levels.

The increased IL-1 and IL-6 could be seen as pointing towards an enhanced immune response. However, this was not the case as it has been shown that 1,25(OH)₂D₃ did inhibit the immune response. The IL-1 and IL-6 could in fact be acting on other cells that were responsive to these cytokines. It is tempting to speculate that while 1,25(OH)₂D₃ was shutting down the immune response it was also responsible for increasing the differentiation of the monocyte/macrophage lineage to form osteoclasts thereby enabling the performance of its recognised function, the mobilisation of calcium. It is also possible that the IL-1 and IL-6 were acting at the level of the bone cells; osteoclasts/osteoblasts.

An attempt was made using purified monocytes as the starting cell population. However as with the PBMC population unstimulated cells also produced significant amounts of IL-1 and IL-6. The IL-6 produced in unstimulated cells can be attributed to the FCS whereas the IL-1 produced under similar conditions must be a consequence of the cell separation procedure.

The role of 1,25(OH)₂D₃ on the activity of other cytokines like IL-1 and IL-6 appears to be negligible. The inhibition of IL-2 by 1,25(OH)₂D₃ is a major phenomenon in comparison to these cytokines.

CHAPTER 6.

1,25 DIHYDROXYVITAMIN D3 AND THE T - CELL SUBSETS.

6.1. THE TARGETS FOR 1,25 DIHYDROXYVITAMIN D₃.

6.1.1. The lymphocyte.

The heterogeneous nature of lymphocytes is well established. There are two classes of lymphocytes each deriving their name from their maturation site. Lymphocytes that are dependent on the thymus for development are called thymus dependent cells or T-cells. Thymus independent lymphocytes include precursors of antibody producing cells and these are termed B-cells. The name arises from the situation in birds. In birds the antibody producing cells are dependent for their development on a gut associated lymphoid organ, the bursa of Fabricius, and this led to term B-cells. However, no bursa equivalent has been found in mammals but the term B-cells is still used to describe lymphocyte precursors of immunoglobulin producing cells throughout immunology. There is a third category of lymphocytes referred to as null cells. The null population includes lymphocytes called killer lymphocytes or K-cells which perform the function of killing some cells that have been sensitised with IgG antibody.

6.1.2. The T-cell.

With the development of antibodies as a research tool it soon became apparent that T-cells themselves are in fact a heterogeneous population. Antibodies to human T-lymphocyte surface determinants demonstrate the existence of at least two functionally distinct subsets, and that these subpopulations express unique cell surface molecules. The two distinct subsets are T helper/inducer cells and T suppressor/cytotoxic cells. T helper/inducer (T_H) are often referred to as either CD4 +ve or T4 +ve cells, the CD4 refers to T-cell determinant present on the membrane (CD, clonal determinant). T suppressor/cytotoxic cells (T_s) are referred to as CD8 +ve or T8 +ve cells. The terminology of the surface antigens were designated at the first international workshop

on leukocyte differentiation antigens. The function of these subsets are very much as their names imply. T_H cells provide helper/inducer function for T - T, T - B and T - macrophage interactions whereas T_s function in a suppressor mode and contain the majority of the cytotoxic T-cell activity.

With the development of more and more refined monoclonal antibodies and technological advancement it has become clear that even these subsets are heterogeneous. However these subsets i.e. T_H and T_s provide a starting point for looking for the target for $1,25(OH)_2D_3$.

6.2. METHODS FOR SUBSET PURIFICATION.

There are two methods that are commonly used to prepare purified subsets of T-cells. The first method and until recently the most popular method is the separation of subsets using plastic petri dishes coated with antibodies against the antigenic determinants (CD4, CD8). This technique is referred to as panning, and can be used for both positive selection and negative selection of the required subset. As mentioned in section 2.2.2. this technique was attempted but unfortunately the yield was unsatisfactory. The second method which is relatively new but is becoming increasingly more popular is to fractionate using an immunomagnetic separation (IMS) technique. The principle of this method is simple, - particles coated with the antibody against the determinant of choice are incubated with the unfractionated cell population. The core of the particles contains deposits of γ ferric oxide (γ Fe₂O₃) and confers superparamagnetic properties which allows a consistent and reproducible reaction to a magnetic field. Binding of the particle to the subset of lymphocytes followed by application of a magnetic field results in a separated subset of lymphocytes. The cells separated by this technique are in the order of 99 % pure and are greater than 95 % viable. The cells are capable of functioning in most biological assays without the need to remove these particles. The particles could be removed if required by incubation overnight at 37 °C in RPMI 1640 + 1 % F.C.S..

6.3. SUBSETS IN LYMPHOCYTE ACTIVATION.

6.3.1. The effect of $1,25(\text{OH})_2\text{D}_3$ on lymphocyte activation - in T_H and T_s .

PBMCs were obtained from eight normal individuals by density gradient centrifugation (section 2.2.1.). The cells were resuspended in PBS + 1 % F.C.S. at a cell density of 3×10^6 cells/ml. The subsets were fractionated using Dynal[®] beads coated with the antibody against the determinants CD4 and CD8 (section 2.2.2.). The cells were counted and resuspended at 6×10^5 cells/ml for the CD4 +ve cells and 3×10^5 cells/ml for the CD8 +ve cells. These concentrations are in keeping with the theoretical concentrations of subsets that would be present in a normal lymphocyte transformation assay. The lymphocyte transformation assay was set up as described in section 2.7. with the modified cell concentrations in the presence of a range of $1,25(\text{OH})_2\text{D}_3$ concentrations. The cells were cultured for a total of 72 hours at 37 °C in a humidified atmosphere of 5 % CO_2/Air . Four hours prior to the end of the incubation period, the cells were pulsed with 1 μCi of tritiated thymidine (3[H]-thy). The cells were harvested and the counts per minute (cpm) of 3[H]-thy incorporated established.

6.3.2. T_H proliferation inhibited by $1,25(\text{OH})_2\text{D}_3$.

The effect of $1,25(\text{OH})_2\text{D}_3$ on each of the subsets (T_H and T_s) stimulated with PHA, Con A and PWM are documented in figure 6.3.1.. It is clear that it was only the T_H cell population that was stimulated under the conditions used in the assay and in the original lymphocyte assay set up with unfractionated PBMCs (section 3.4.2.). The T_s cell population was not stimulated by any of the mitogens. There was therefore no apparent inhibition of lymphocyte activation by $1,25(\text{OH})_2\text{D}_3$. The T_H cell populations were significantly inhibited by $1,25(\text{OH})_2\text{D}_3$ whether the cells were activated by PHA, Con A or PWM. PHA stimulated T_H cells were significantly inhibited by 10^{-10} M $1,25(\text{OH})_2\text{D}_3$ whereas Con A and PWM stimulated cells were significantly inhibited by 10^{-13} M

The effect of 1,25 dihydroxyvitamin D3 on the proliferation of PHA, Con A and PWM induced cell subsets (TH and Ts)

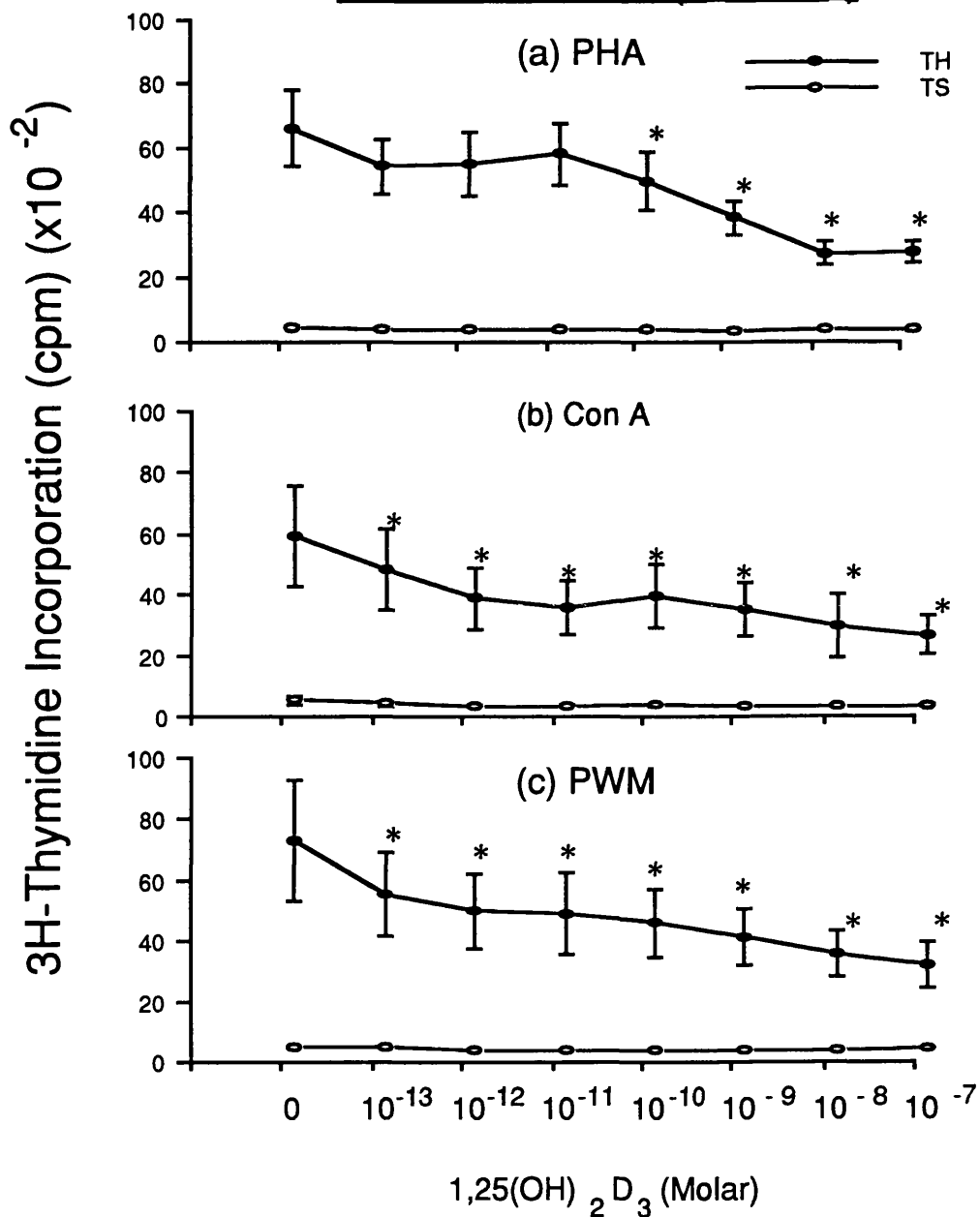


FIGURE 6.3.1.

The figure represents the proliferation of the two cell subsets stimulated with either PHA, Con A and PWM and cultured in the presence of 1,25 dihydroxyvitamin D3. The data are presented as the mean of 8 normal individuals and the error bars are the standard error of the mean (SEM). The statistical test carried out on the data was the paired Wilcoxon test test (*p < 0.05) comparing 0 to all other concentrations of 1,25 dihydroxyvitamin D3.

1,25(OH)₂D₃. The proliferation obtained in these experiments with purified cell subsets was a degree of magnitude lower than when unfractionated cells were used. The reason for this are twofold ; the actual number of cells at the start of the culture were lower (although the number of T_H were similar) and secondly there was a lack of accessory cells which could function to amplify the lymphocyte activation response.

6.3.3. Inhibition of proliferation - mediated via T_H cells.

The observation that the T_H cells activated with PHA were significantly inhibited by 1,25(OH)₂D₃ at 10⁻¹⁰ M correlates well with the effects on unfractionated PBMCs (section 3.4.2.). This would then argue that the effect seen in unfractionated PBMCs is due to an effect on the T_H cell population.

The data obtained for the effects of 1,25(OH)₂D₃ on Con A and PWM induced proliferation on T_H cells are a little more difficult to explain. However, by definition a mitogen promotes T-cell proliferation which would always require some activation of T_H cells. It is therefore possible that the T_H cells activated by these mitogens were the subpopulation of cells specifically targeted by the 1,25(OH)₂D₃ hence the reason significance was attained at lower concentrations (10⁻¹³ M). PHA on the other hand activates all T_H cells and therefore the inhibition takes more 1,25(OH)₂D₃ to show up as significant.

What is apparent from these results is that 1,25(OH)₂D₃ does not appear to mediate its inhibitory effect through cells of the cytotoxic/suppressor class. If 1,25(OH)₂D₃ was to mediate the inhibitory effect through the T_s cell population then there would have been no comparable inhibition observed in the T_H cell population. However an inhibition was observed and this inhibition was comparable percentage wise with the results obtained from unfractionated cells. It is therefore possible to rule out the postulate that 1,25(OH)₂D₃ mediates its effect via the differentiation of suppressor cells. The observation that 1,25(OH)₂D₃ does not lead to an increase in T_s cells strengthens this particular argument.

6.4. SUBSETS AND INTERLEUKIN - 2 ACTIVITY.

6.4.1. The effects of 1,25(OH)₂D₃ on IL-2 activity - T_H and T_s.

PBMCs were obtained from eight normal individuals by density gradient centrifugation (section 2.2.1.). The cells were resuspended in PBS + 1 % F.C.S.. The resuspended cells were fractionated to yield the T-cell subsets (section 2.2.2.). The cells were counted and resuspended in complete RPMI 1640 + 1 % F.C.S. at 6×10^5 cells/ml for the T_H cells and 3×10^5 cells/ml for the T_s cells. The cells were then stimulated with either PHA (1 µg/ml, Wellcome Grade) or Con A (5 µg/ml) in the presence of a range of 1,25(OH)₂D₃ concentrations. The cells were cultured at 37 °C in a humidified atmosphere of 5 % CO₂/Air for 48 hours following which the IL-2 containing supernatants were harvested. The IL-2 activity was assessed in the HT-2 bioassay (section 2.8.2.).

6.4.2. As expected only T_H produce IL-2 activity.

The IL-2 activity in the presence of 1,25(OH)₂D₃ obtained in each of the subsets is presented in tables 6.4.1. and 6.4.2.. The results are expressed as the mean with the standard error in parenthesis. It is clear that 1,25(OH)₂D₃ significantly inhibits IL-2 activity in T_H cells and that this inhibition can be observed in cells stimulated with either PHA or Con A. The T_s cells as expected did not produce any IL-2 activity when stimulated with Con A and therefore 1,25(OH)₂D₃ did not affect these cells. The T_s cells stimulated with PHA did produce a small amount of IL-2 activity and 1,25(OH)₂D₃ did inhibit this activity although no significance was attained. The IL-2 activity in these cells was probably due to contaminating T_H in some of the preparations as this activity was not uniformly found in all the individuals. The data are also represented graphically in figure 6.4.1., standard errors of the mean appear excessive, but the actual IL-2 activity was low and this may account for the large distribution of the results. However, the actual inhibition in terms of percentage was comparable to the inhibition observed in unfractionated cells i.e. at 10⁻¹¹ M there was inhibition in excess of 50 % (figure 6.4.2.).

IL-2 activity in T-Helper cells cultured in the presence of
1,25 dihydroxyvitamin D3 and stimulated with either PHA or Con A

Mitogen	1,25(OH) ₂ D ₃ (Molar)	IL-2 Activity (units/ml)
PHA	0	3.55 (1.40)
PHA	10 ⁻¹³	3.06 (1.24)
PHA	10 ⁻¹²	3.12 (1.42)
PHA	10 ⁻¹¹	2.92 (1.50)
PHA	10 ⁻¹⁰	2.86 (1.53)
PHA	10 ⁻⁹	2.39* (1.18)
PHA	10 ⁻⁸	2.00* (0.97)
PHA	10 ⁻⁷	1.62* (0.85)
Con A	0	5.34 (2.99)
Con A	10 ⁻¹⁰	4.09* (2.66)

TABLE 6.4.1.

The figure presents the IL-2 activity in TH cells stimulated with PHA and TS cells stimulated with Con A, both subsets were cultured in the presence of 1,25 dihydroxyvitamin D3. The IL-2 activity was expressed as units/ml. The data are presented as the mean of 8 normal individuals with the standard error of the mean represented as error bars. Statistics in the form of a Wilcoxon test were carried out (* p < 0.05) comparing 0 with each of the concentrations of 1,25(OH)₂D₃ tested for either mitogen.

IL-2 activity in T-Suppressor cells cultured in the presence of
1,25 dihydroxyvitamin D3 and stimulated with either Con A or PHA

Mitogen	1,25(OH) ₂ D ₃ (Molar)	IL-2 Activity (units/ml)
Con A	0	1.64 (0.80)
Con A	10 ⁻¹³	1.29 (0.64)
Con A	10 ⁻¹²	1.48 (0.72)
Con A	10 ⁻¹¹	1.03 (0.50)
Con A	10 ⁻¹⁰	1.46 (0.75)
Con A	10 ⁻⁹	1.40* (0.70)
Con A	10 ⁻⁸	1.50 (0.74)
Con A	10 ⁻⁷	0.94 (0.48)
PHA	0	1.26 (0.45)
PHA	10 ⁻¹⁰	1.05 (0.45)

TABLE 6.4.2.

The table presents the IL-2 activity of Ts cells stimulated with either PHA or Con A and cultured in the presence of 1,25 dihydroxyvitamin D3. The data were obtained from 8 normal individuals. The data are the mean with the standard error of the mean in parenthesis. The data were subjected to the Wilcoxon test ((* p < 0.05) comparing 0 to each of the concentrations of 1,25(OH)₂D₃ tested and stimulated with either mitogen.

The IL-2 activity present in supernatants obtained from TH and Ts stimulated with PHA and Con A respectively and cultured in the presence of 1,25 dihydroxyvitamin D3

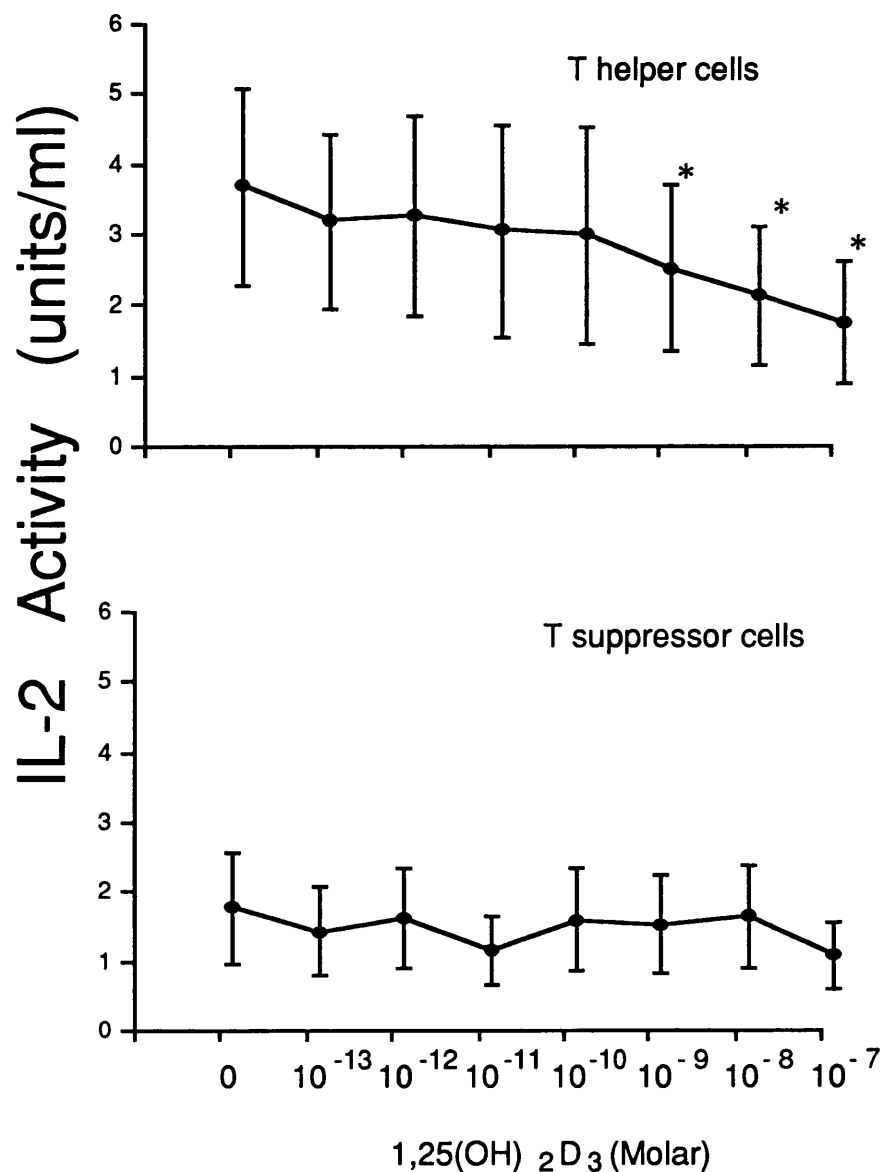


Figure 6.4.1.

The figure represents the IL-2 activity in TH cells stimulated with PHA and Ts cells stimulated with Con A, both subsets were cultured in the presence of 1,25 dihydroxyvitamin D3. The IL-2 activity was expressed as units/ml. The data are presented as the mean of 8 normal individuals with the standard error of the mean (SEM) represented as error bars. Statistics in the form of a paired Wilcoxon test were carried out (*p <0.05) comparing 0 with all other concentrations of 1,25 dihydroxyvitamin D3.

The IL-2 activity present in supernatants obtained from TH cells stimulated with PHA and cultured in the presence of 1,25 dihydroxyvitamin D3 expressed as a percentage

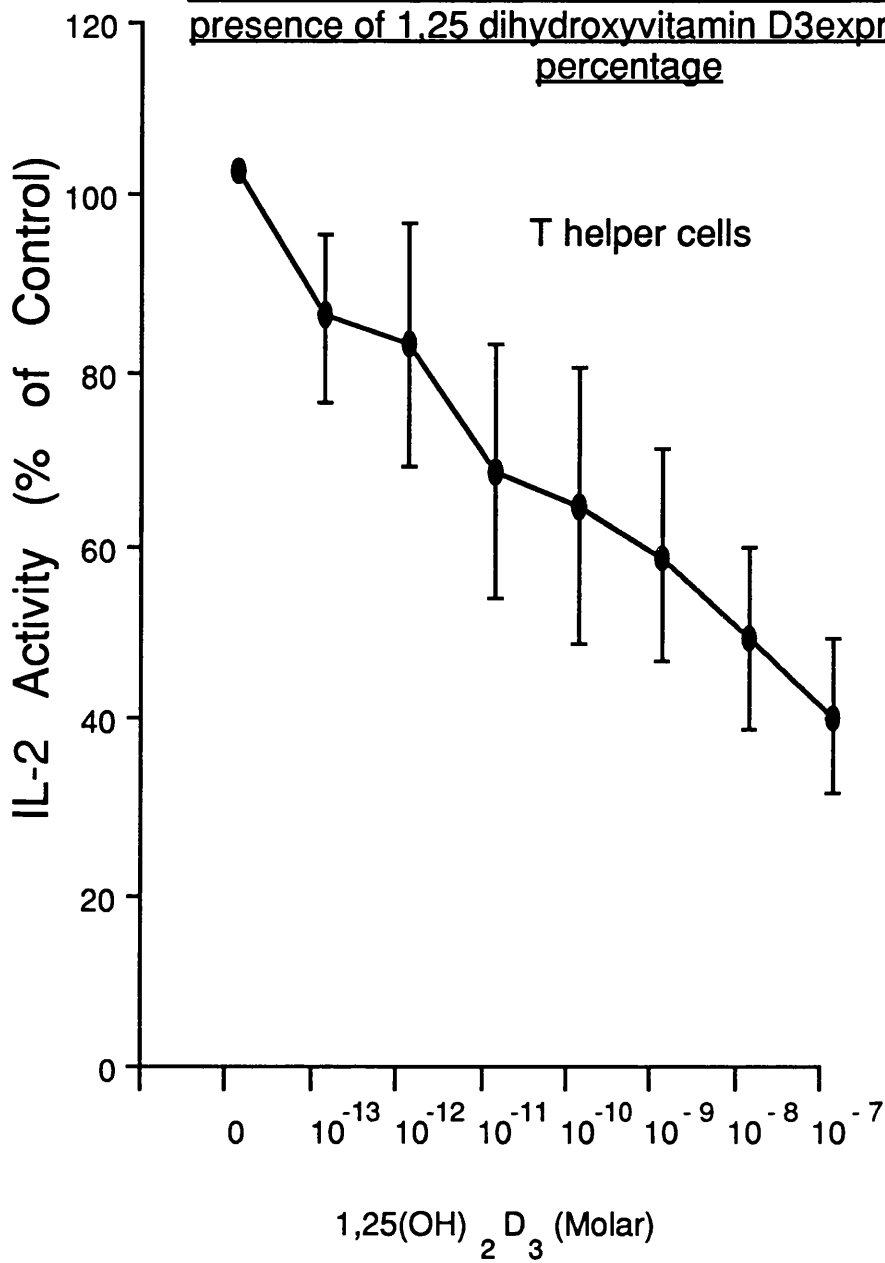


FIGURE 6.4.2.

The figure represents the IL-2 activity in TH cells stimulated with PHA and cultured in the presence of 1,25 dihydroxyvitamin D3. The IL-2 activity was expressed as the percentage of control. The data are presented as the mean of 8 normal individuals with the standard error of the mean (SEM) represented as error bars.

The low IL-2 activity observed in the T_H cells could be the result of low cell number or could be due to the lack of accessory factors normally provided by the depleted cells. However, what is clear is that 1,25(OH)₂D₃ appears to act on the T_H cells and not the T_s cells when mediating its inhibition.

6.5. INHIBITION BY 1,25(OH)₂D₃ WAS NOT EXPLAINED BY INCREASED DIFFERENTIATION OF T_s.

The promotion of differentiation by 1,25(OH)₂D₃ is an established phenomenon. The discovery of the vitamin D receptor (VDR) in some tumour lines raised the possibility that the active D metabolite may effect cell differentiation. Physiological concentrations of 1,25(OH)₂D₃ (10⁻¹¹ M - 10⁻¹⁰ M) were shown to induce differentiation of both murine and human myloid leukaemic cells *in vitro*.

It was postulated by Tsoukas et al that the inhibition of PBMCs by 1,25(OH)₂D₃ in terms of both proliferation and of IL-2 activity may be mediated by promotion of differentiation of suppressor cells. At the time this was a perfectly feasible proposition. However, the data presented in chapter 3 and 4 and in this present chapter argue against the involvement of suppressor cells in this response. In chapter 3 the inhibition of proliferation stimulated with Con A and with PHA by 1,25(OH)₂D₃ suggested that the T_H cell mitogen and the T_s cell mitogen were both similarly affected. The Con A stimulated cells requiring slightly more 1,25(OH)₂D₃ to achieve significance. In chapter 4 it was demonstrated that 1,25(OH)₂D₃ inhibited IL-2 activity and that this could account for the inhibition of lymphocyte proliferation. The present chapter has attempted to investigate the importance of the subsets T_H and T_s in the inhibition of both these immune functions by 1,25(OH)₂D₃ and to establish whether or not suppressor cells have a role to play in the mechanism. It is clear from the data that the inhibition by 1,25(OH)₂D₃ is restricted to the T_H cells as far as lymphocyte activation and IL-2 activity is concerned. It is therefore possible to assume that the T_s cells are not involved in mediating the 1,25(OH)₂D₃ effect.

The observation that the stimulation of T_H cells by Con A achieves significance at 10⁻¹³ M 1,25(OH)₂D₃ is interesting bearing in mind that the proliferation of unfractionated cells stimulated with Con A does not achieve significance until 10⁻⁹ M. A possible explanation for this difference could be that whereas PHA acts as a general T_H cell mitogen, Con A may stimulate its suppressor cells by way of a specific population of T_H cells which is particularly sensitive to 1,25(OH)₂D₃. The same could be said for stimulation with PWM. In the unfractionated cells stimulated with Con A and PWM the effect of 1,25(OH)₂D₃ on this specific population would be swamped by proliferation of other cell populations. It may be that this subpopulation of T_H cells is the actual target for 1,25(OH)₂D₃.

CHAPTER 7.

1,25 DIHYDROXYVITAMIN D3 AND THE SOLUBLE INTERLEUKIN - 2 RECEPTOR.

7.1. INTERLEUKIN - 2 RECEPTOR.

As with polypeptide hormones and other growth factors, the mode of action of interleukin-2 (IL-2) is mediated via binding to a specific cell surface receptor on responsive T-cells. Also like other hormone receptor interactions the binding of IL-2 to its receptor is characteristically a high affinity binding event. The IL-2 receptor (IL-2R) conforms to the definition of a true hormone receptor molecule with regard to the following criteria ; high affinity, saturability, ligand specificity and target cell specificity (Robb, 1981). These criteria were demonstrated by the use of biosynthetically radiolabelled IL-2. More significantly it has been demonstrated that the concentrations of IL-2 that bind with high affinity to IL-2R are the same as the concentrations of IL-2 that promote T-cell proliferation. In the first instance it was thought that only T-cells stimulated with mitogenic factors were responsive to IL-2 however, it was later revealed that B-cells, Natural Killer cells (NK cells) and Lymphocyte activated killer cells (LAK cells) were also responsive (Mizel, 1989). The ability of cells to respond to IL-2 is intimately associated with the expression of the IL-2 receptor. Resting T-cells do not express the form of IL-2R required for the transmission of a proliferative signal. T-cells become responsive to IL-2 in the presence of antigen and a presenting cell e.g. a macrophage and an appropriate antigen.

7.1.2. The structure.

Initially the structure of the IL-2 receptor proved elusive. The first monoclonal antibody against the IL-2 receptor was developed by Uchiyama et al (Uchiyama, 1981). A problem occurred when it was discovered that this antibody reacted with significantly more cell surface molecules than could be detected by high affinity radiolabelled IL-2 (Leonard, 1982). In following up this observation it was shown that the excess cell surface molecules did in fact bind IL-2 albeit at a lower affinity than the high affinity IL-2 receptor (Robb, 1984). The cDNA isolated using this monoclonal antibody revealed that the product of this cDNA did bind IL-2 but only at a low affinity (Sabe, 1984 and Greene, 1985). It was later established that authentic high affinity IL-2 receptors consisted of two distinct polypeptide chains each containing an IL-2 binding site (Tsudō, 1986 and Teshigawara, 1987). These two polypeptide chains were designated the p75 chain (75kD) and the p55 chain (55 kD) and assigned either α or β depending on which convention was used (first discovered v's highest molecular weight).

The identification and isolation of a leukaemic cell line that expressed only the p75 chain made it possible to look at the distinct IL-2 binding characteristics of the two separate chains (Tsudō, 1986 and Teshigawara, 1987). It was established that the interaction between the two chains was not via a covalent disulphide bond. It appears that the chains interacted via non covalent forces to form a heterodimeric high affinity IL-2 receptor (Smith, 1988). It also emerged that the binding site for the p75 chain was separate and distinct from the p55 chain (figure 7.1.1.). If the chains were studied independently the p55 chain has a dissociation constant (K_D) of 10^{-8} M (low affinity) whereas the p75 chain has a K_D of 10^{-9} M (intermediate affinity) (Teshigawara, 1987). As a consequence of when both chains were expressed together in the same cell a receptor with a dissociation constant (K_D) of 10^{-11} M (high affinity) was formed. These observations argue for the individual chains recognising different residues on the IL-2 molecule as there was evidently no competition for the IL-2.

A diagrammatic representation of the high affinity
Interleukin - 2 receptor(IL-2R)

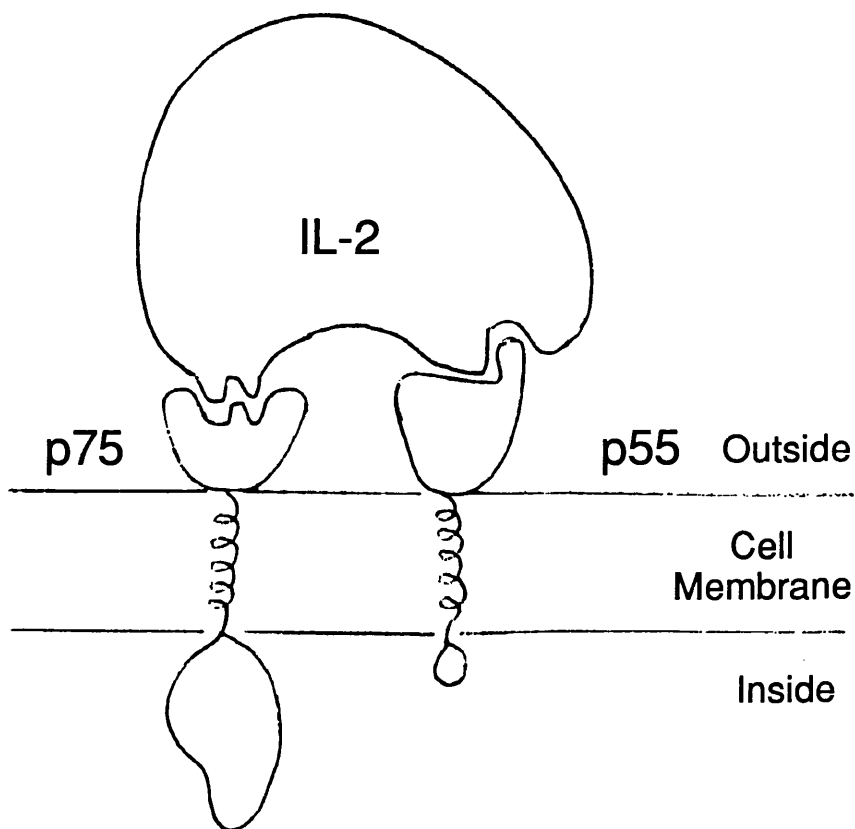


Figure 7.1.1.

The figure represents the high affinity IL-2R. The two chains, p75 and p55, present in the high affinity receptor bind IL-2 independently but also cooperate. The two chains are not covalently linked by disulphide bonds. The p75 chain binds IL-2 with an intermediate affinity and is constitutively expressed. The p75 chain is responsible for signal internalisation hence the larger cytoplasmic domain. The p55 (or Tac) is a low affinity receptor which is induced after cell activation.

Instead, cooperation between the binding sites leads to a 100 - to 1000 - fold increase in the binding affinity of the heterodimer. The kinetics and the equilibrium constants of the individual chains were very different but when combined enhance the high affinity binding. IL-2 binds to and dissociates from the p75 chain very slowly whereas the IL-2 reacts rapidly with the p55 chain. The high affinity receptor possesses a fast association rate derived from the fast acting p55 chain and a slow dissociation rate derived from the slow acting p75 chain. The kinetics in this situation combine to enhance the affinity of the receptor.

7.2. THE SOLUBLE INTERLEUKIN - 2 RECEPTOR.

The extensive study of interleukin-2 and its interaction with its receptor led to the detection of a fully soluble form of the IL-2 receptor in culture supernatants (Rubin, 1985). The identification and quantitation of this soluble IL-2 receptor (sIL-2R) was made possible by the development of a sandwich enzyme linked immunoabsorbant assay (ELISA) (Rubin, 1985). This ELISA employed two noncompetitive anti-IL-2R monoclonal antibodies. It soon became evident that the sIL-2R was present at low levels in sera from normal individuals and at elevated levels in sera from patients with various neoplastic disorders. It was apparent that this parameter was useful as a means of assessing immune function.

The sIL-2R protein was dependent on activation of the immune system. The glycosylated protein was fully soluble with a molecular weight of approximately 45 kD (Rubin, 1985, Rubin, 1986 and Hakimi 1987) and was probably generated by the proteolytic cleavage of the cell surface Tac protein (also referred to as p55) (Robb, 1987, Loughnan, 1988 and Meidel, 1988). This was made more likely by the observation that there does not appear to be any evidence for an alternatively spliced Tac mRNA transcript. The binding affinity of the sIL-2R was on a par with the p55 chain. In certain circumstances the sIL-2R concentration has been elevated to such an extent that the IL-2 function was coincidentally impaired suggesting that the sIL-2R may act as an antagonist of IL-2 mediated responses. It was to this end that the effects of 1,25(OH)₂D₃ on sIL-2R concentration were studied.

7.2.1. sIL-2R expression in the presence of 1,25(OH)₂D₃.

PBMCs were obtained from seven normal individuals by density gradient centrifugation (section 2.2.1.). The cells were resuspended at 10⁶ cells/ml in complete RPMI 1640 + 2 % F.C.S. and stimulated with 1 µg/ml PHA (Wellcome Grade) in the presence of a range of 1,25(OH)₂D₃ concentrations (10⁻¹³ M - 10⁻⁷ M). The cells were cultured for 48 hours at 37 °C in a humidified atmosphere of 5 % CO₂/Air. The supernatants were harvested and stored at -20 °C until assayed for both IL-2 activity and cell-free sIL-2R.

The IL-2 activity was assessed using the HT-2 bioassay (section 2.8.2.) and the sIL-2R was assessed using the commercially available Cellfree interleukin-2 receptor test kit (T-cell Sciences, Inc.; section 2.11.2.).

7.2.2. sIL-2R concentration increases as IL-2 activity decreases.

The IL-2 activity and the sIL-2R concentration for each of the concentrations of 1,25(OH)₂D₃ are presented in table 7.2.1.. It is clear that while 1,25(OH)₂D₃ inhibits IL-2 activity, 1,25(OH)₂D₃ at the higher concentrations (greater than 10⁻¹¹ M) increases the concentration of sIL-2R in the supernatants. The data are also depicted in figure 7.2.1. and provide a clear demonstration of an all or nothing response of the sIL-2R. The concentration of sIL-2R appears to go along at a constant level between control and 10⁻¹¹ M 1,25(OH)₂D₃ and then between 10⁻¹¹ M and 10⁻¹⁰ M (interestingly the physiological range) the amount of sIL-2R increases and maintains a plateau at this higher level. This increase in sIL-2R is statistically significant. On the other hand, the IL-2 activity was inhibited from the outset and becomes statistically significant at 10⁻¹² M.

The effect of 1,25 dihydroxyvitamin D₃ on both IL-2
activity and the amount of soluble IL-2 receptor

1,25(OH) ₂ D ₃ (Molar)	IL-2 Activity (units/ml)	soluble IL-2 receptor (units/ml)
0	30.0 (5.4)	980.0 (129.9)
10 ⁻¹³	26.7*	1010.0 (101.5)
10 ⁻¹²	24.0* (4.9)	967.1 (104.5)
10 ⁻¹¹	19.0* (4.1)	942.1 (97.4)
10 ⁻¹⁰	20.4* (3.9)	1422.1* (225.4)
10 ⁻⁹	16.7* (3.2)	1395.7* (177.3)
10 ⁻⁸	12.9* (2.3)	1348.6* (275.5)
10 ⁻⁷	12.6* (2.2)	1435.0* (252.5)

Table 7.2.1.

The table presents the IL-2 activity and the concentration of soluble IL-2 receptor in the supernatant of cells stimulated with PHA and cultured in the presence of 1,25 dihydroxyvitamin D₃. The data are presented as the mean of 7 normal individuals and the standard error of the mean are presented in parenthesis. Statistics in the form of a paired Wilcoxon test was performed on the data (* p > 0.05) comparing 0 with all other concentrations of 1,25(OH)₂D₃.

The effect of 1,25 dihydroxyvitamin D₃ on the IL-2 activity and on the soluble IL-2 receptor

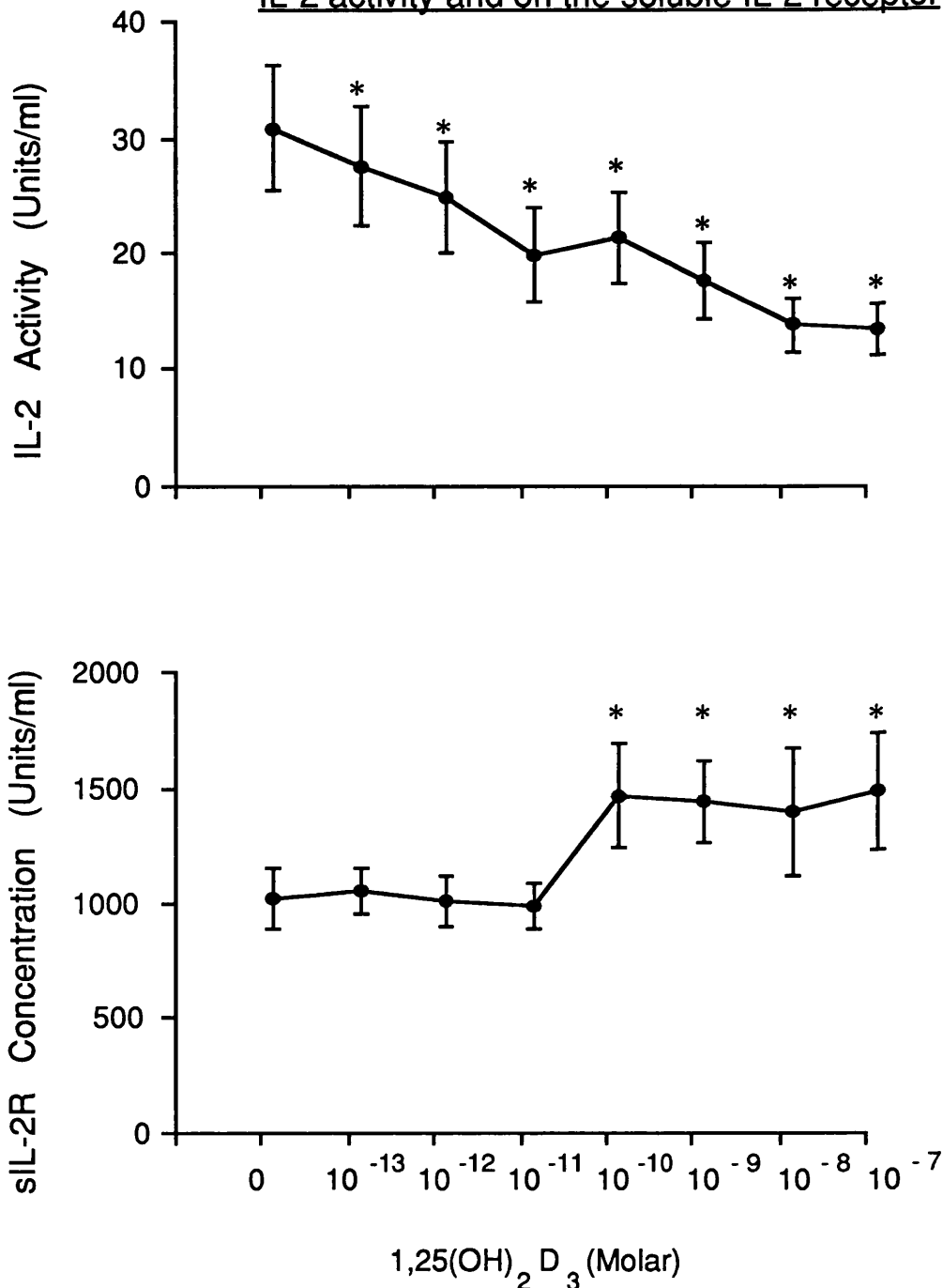


FIGURE 7.2.1.

The figure represents the IL-2 activity and also the concentration of soluble IL-2 receptor in the supernatant of cells stimulated with PHA and cultured in the presence of 1,25 dihydroxyvitamin D₃. The data are presented as the mean of 7 normal individuals and the standard error of the mean are represented as error bars. Statistics in the form of a paired Wilcoxon test was performed on the data (* p > 0.05) comparing 0 to all other concentrations of 1,25 dihydroxyvitamin D₃.

7.2.3. Increased sIL-2R occurs over the physiological range.

These observations imply that $1,25(\text{OH})_2\text{D}_3$ over the physiological range (10^{-11} M - 10^{-10} M) has a profound effect on the concentration of sIL-2R. Over this range the sIL-2R concentration was significantly increased. The implication is that $1,25(\text{OH})_2\text{D}_3$ increases the proteolytic cleavage of the high affinity IL-2 receptor and that the sIL-2R absorbs the IL-2 and consequently acts as an antagonist for IL-2 mediated responses. The IL-2 activity is inhibited throughout the range of $1,25(\text{OH})_2\text{D}_3$ - not just at concentrations above 10^{-11} M - which implies that there is was more than one mechanism at work in this system.

The data presented in chapters three and four regarding the effects of sex hormones on both lymphocyte activation and IL-2 activity should be considered at this juncture. In these chapters significant inhibitions of lymphocyte activation and IL-2 activity by the sex hormones were noted at 10^{-10} M but the values returned to control levels at the higher concentrations. These data also hinted at the existence of two mechanisms. It was conceivable that $1,25(\text{OH})_2\text{D}_3$ was acting as a classical steroid hormone up to and including 10^{-11} M and for the remainder of the range of $1,25(\text{OH})_2\text{D}_3$ concentrations (10^{-10} M - 10^{-7} M) the inhibition was occurring via changes in the sIL-2R concentrations. This implies that the initial inhibition was a result of classical genomic changes brought about by steroid hormones and that $1,25(\text{OH})_2\text{D}_3$ had a unique contribution to make at concentrations in excess of 10^{-11} M .

CHAPTER 8.

THE EFFECT OF $1,25(\text{OH})_2\text{D}_3$ ON SELECTED mRNAs.

8.1. THE EFFECT OF 1,25(OH)₂D₃ ON mRNAs FOR IL-2 AND IL-2R.

1,25(OH)₂D₃ was thought to act as a steroid hormone in the immune system. The first action of a steroid hormone is accepted as a change in the message going to the protein assembly apparatus. This chapter deals with the effect of 1,25(OH)₂D₃ on the mRNA for IL-2 and on the mRNA for the IL-2 receptor.

8.1.1. The effect of 1,25(OH)₂D₃ on mRNA for IL-2 and IL-2R at four hours.

PBMCs were obtained from a normal individual by density gradient centrifugation over Ficoll - hypaque (see section 2.2.1.). The cells were resuspended at a cell density of 10⁶ cells/ml in complete RPMI 1640 + 2 % FCS and stimulated with 1 µg/ml PHA (Wellcome) in the presence of either vehicle (ethanol), 10⁻¹⁰ M or 10⁻⁷ M 1,25(OH)₂D₃. The cells were cultured for 4 hours at 37 °C in a humidified atmosphere of 5 % CO₂/Air. The cells were harvested and washed in PBS. The RNA was extracted from the cells using an acid guanidinium thiocyanate - phenol - chloroform extraction (see section 2.12.1). The mRNA was assessed (see section 2.12.2.) and characterised on a Northern blot analysis. The samples were run on a 1 % agarose/formamide gel. Each sample was run in three different positions in the gel, such that the gel could be cut in three resulting in three gels each possessing one of each of the samples. The RNA was transferred to the nitrocellulose (see section 2.12.3.). The three blots were probed with either an IL-2 probe, an IL-2R probe or the abl probe. The probes were labelled by an end labelling reaction (see section 2.12.3). The probes were hybridised to the blots and autoradiographs were developed.

The effect of 1,25 Dihydroxyvitamin D3 on the mRNA for

IL-2 and IL-2R after a 4 hour culture

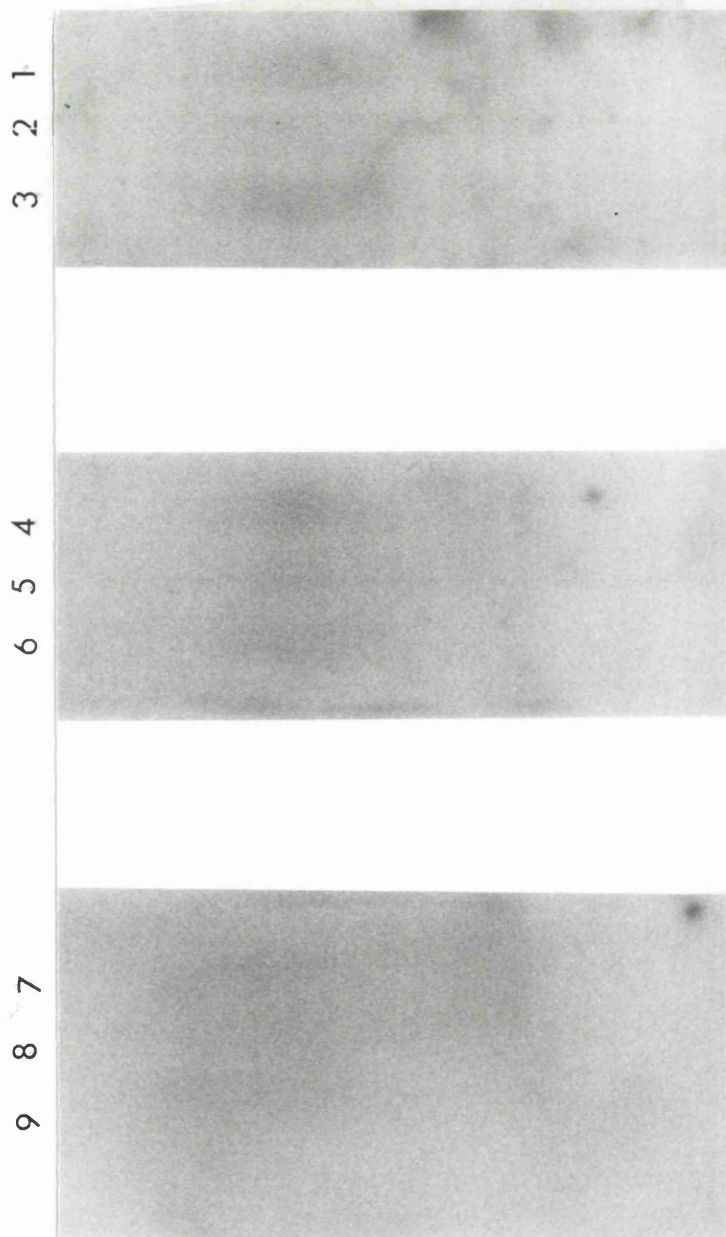


Figure 8.1.1.

The amounts of mRNA complementary to IL-2, IL-2R and abl in PBMCs stimulated with PHA and cultured in the presence of either 10^{-10} M or 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ or in the presence of ethanol (the carrier) are represented. The mRNA in lanes 1, 4 and 7 were derived from cells cultured in the presence of ethanol, the mRNA in lanes 2, 5 and 8 were derived from cells cultured in the presence of 10^{-10} M $1,25(\text{OH})_2\text{D}_3$ and the mRNA in lanes 3, 6 and 9 were derived from cells cultured in the presence of 10^{-7} M $1,25(\text{OH})_2\text{D}_3$. Lanes 1 to 3 were probed with IL-2, lanes 4 to 6 were probed with IL-2R and lanes 7 to 9 were probed with abl. The figure reveals little but should be compared with figure 8.2.1..

8.1.2. 1,25(OH)₂D₃ has little effect on IL-2 and IL-2R at four hours.

The autoradiograph depicted in figure 8.1.1. shows the amount of specific RNA for each of the three probes. Lanes 1 to 3 represent the amount of RNA specific for the IL-2 message. Lane 1 was RNA from cells cultured in the presence of ethanol, lane 2 was RNA from cells cultured in the presence of 10^{-10} M 1,25(OH)₂D₃ whereas lane 3 was RNA from cells cultured in the presence of 10^{-7} M 1,25(OH)₂D₃. There was no apparent difference in the amount of IL-2 message in each of these conditions. Lanes 4 to 6 were similarly set up and this time these lanes were a measure of the expression of the IL-2 receptor. Again, there appeared to be no difference in the amount of message for IL-2R whether the cells were cultured in the presence of vitamin D or not. Lane 7 to 9 were also set up in the same fashion but were probed with the abl probe. This is a house keeping gene and so should be present in the same amounts if the amount of RNA loaded onto each lane was the same. The autoradiograph is not very distinctive, the best that can be said is that at 4 hours the cells are not expressing much mRNA for either IL-2 or IL-2R. The amount of RNA loaded onto the gel was similar in each lane as assessed by the preparation gel. The blot hybridised to the abl gene however did not show very much RNA on the blots. An explanation therefore could be that the amount of RNA loaded onto the cell was inadequate or alternatively that the hybridisation conditions were too stringent. This set of results were therefore inconclusive.

8.2. THE EFFECT OF 1,25(OH)₂D₃ ON mRNA FOR IL-2 AND IL-2R AFTER A LONGER INCUBATION.

It remained possible that the 1,25(OH)₂D₃ was inhibiting the IL-2 or IL-2R mRNA at a later stage i.e. there was no effect in the first four hours of culture. The time scale for the expression of vitamin D receptors after stimulation made it possible that an inhibition at this level would occur at a later time point.

8.2.1. The effect of 1,25(OH)₂D₃ on mRNA for IL-2 and IL-2R at twenty hours.

PBMCs were obtained from a normal individual by density gradient centrifugation over a Ficoll - hypaque cushion (see section 2.2.1.). The cells were set up in culture at 10⁶ cells/ml in complete RPMI 1640 supplemented with 2 % FCS and stimulated with 1 µg/ml PHA. The cells were cultured in the presence of either ethanol, 10⁻¹⁰ M 1,25(OH)₂D₃ or 10⁻⁷M 1,25(OH)₂D₃. The cultures were incubated at 37 °C in a humidified atmosphere of 5 % CO₂/Air for 20 hours. At the end of the culture period the cells were pelleted and the RNA was extracted using the acid phenol thiocyanate chloroform method (see section 2.12.2). The mRNA was assessed (see section 2.12.2.) and characterised on a Northern blot analysis. The samples were run on a 1 % agarose/formamide gel. Each sample was run in two different positions in the gel, such that the gel could be halved resulting in two gels each possessing one of each of the samples. The RNA was transferred to the nitrocellulose (see section 2.12.3.). The two blots were probed with either the IL-2 probe or the IL-2R probe. The probes were labelled by an end labelling reaction (see section 2.12.3). The probes were hybridised to the blots and autoradiographs were developed.

The effect of 1,25 Dihydroxyvitamin D₃ on the mRNA for
IL-2 and IL-2R after a 20 hour culture

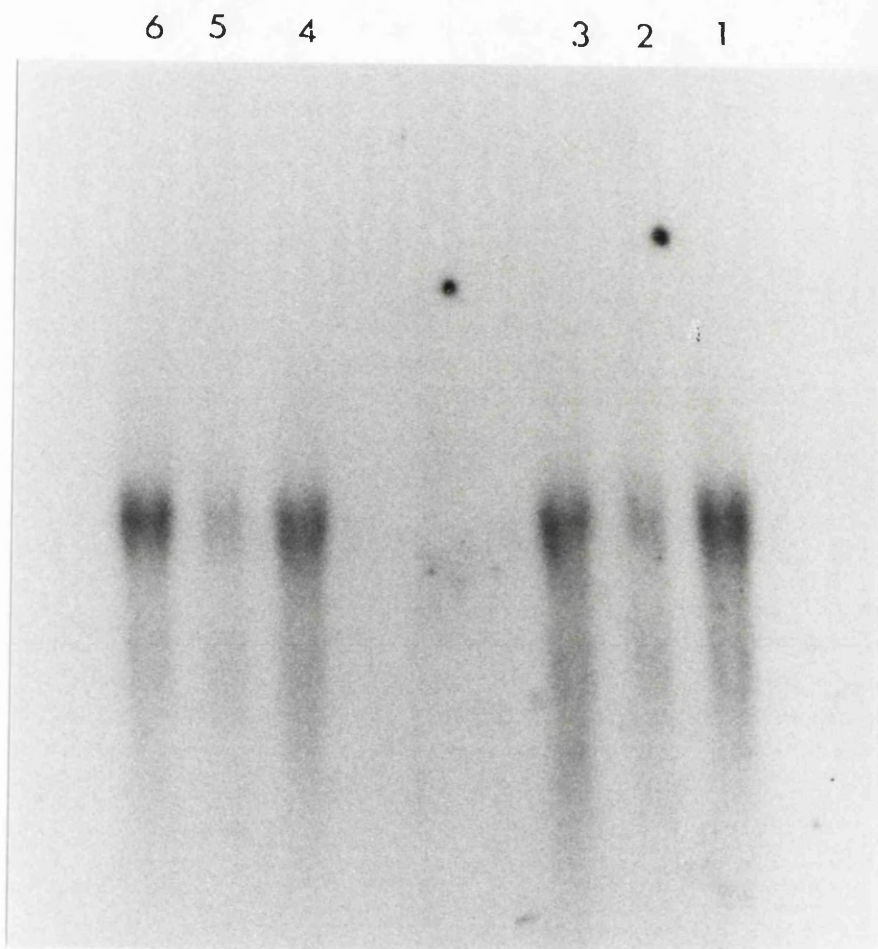


Figure 8.2.1.

The amounts of mRNA complementary to both IL-2 and IL-2R in PBMCs stimulated with PHA and cultured in the presence of either 10^{-10} M or 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ or in the presence of ethanol (the carrier) are represented. The mRNA in lanes 1 and 4 were derived from cells cultured in the presence of ethanol, the mRNA in lanes 2 and 5 were derived from cells in cultured in the presence of 10^{-10} M $1,25(\text{OH})_2\text{D}_3$ and the mRNA in lanes 3 and 6 were derived from cells cultured in the presence of 10^{-7} M $1,25(\text{OH})_2\text{D}_3$. Lanes 1 to 3 were probed with IL-2R and lanes 4 to 6 were probed with IL-2. The figure clearly depicts an inhibition of both IL-2 and IL-2R at 10^{-10} M $1,25(\text{OH})_2\text{D}_3$ and loss of inhibition at 10^{-7} M $1,25(\text{OH})_2\text{D}_3$.

8.2.2. Inhibition by 1,25(OH)₂D₃ followed by recovery.

The autoradiograph shown in figure 8.2.1. depicts the amount of mRNA complementary to IL-2 and IL-2R. The mRNA was obtained from cells cultured in the presence of 1,25(OH)₂D₃ for 20 hours. Lanes 1 to 3 were probed with IL-2R and lanes 4 to 6 were probed with IL-2. Lane 1 contains the mRNA obtained from cells cultured in the presence of ethanol, lane 2 contains the mRNA from cells cultured in the presence of 10⁻¹⁰ M 1,25(OH)₂D₃ and lane 3 contains mRNA from cells cultured in the presence of 10⁻⁷ M 1,25(OH)₂D₃. Lane 1 and lane 3 appear to be of similar intensity so the amount of message for IL-2R can be described as similar in cells cultured in the presence of ethanol and in the presence of 10⁻⁷ M 1,25(OH)₂D₃. Lane 2 is less intense and clearly the message for IL-2R is inhibited by 10⁻¹⁰ M 1,25(OH)₂D₃. Lane 4 contains mRNA from cells cultured in the presence of ethanol, lane 5 contains mRNA from cells cultured in the presence of 10⁻¹⁰ M 1,25(OH)₂D₃ and lane 6 contains mRNA from cells cultured in the presence of 10⁻⁷ M 1,25(OH)₂D₃. Again lanes 4 and 6 appear to be of similar intensity, this implies that there was no difference in the amount of message for IL-2 in cells cultured in the presence of either ethanol or 10⁻⁷ M 1,25(OH)₂D₃. Lane 5 appears to be less intense which would imply that the message for IL-2 is also inhibited by 10⁻¹⁰ M 1,25(OH)₂D₃.

8.2.3. 1,25(OH)₂D₃ inhibits the message for both IL-2 and IL-2R.

At 10⁻¹⁰ M, 1,25(OH)₂D₃ inhibits both the IL-2 and IL-2R directly and this accounts for the inhibition of IL-2 activity documented in chapter 4. It is interesting to note that the amount of message returns to normal levels at 10⁻⁷ M 1,25(OH)₂D₃. It is therefore possible to conclude that the inhibition of IL-2 activity by 1,25(OH)₂D₃ is not wholly explained by an inhibition of the message for IL-2. Clearly the expression of the IL-2R gene is closely linked to the IL-2 gene in that the expression follows the same pattern.

8.3. THE INHIBITION OF IL-2 ACTIVITY BY 1,25(OH)₂D₃ - TWO MECHANISMS.

These results presented in section 8.2. and section 7.2. strengthen the argument for a second mechanism in the inhibition of IL-2 activity by 1,25(OH)₂D₃ speculated upon and mentioned previously. Clearly the effect on the message does not explain the sustained inhibition of IL-2 activity exhibited throughout the range of 1,25(OH)₂D₃ concentrations tested. It is tempting to speculate that over the range 0 to 10⁻¹⁰ M 1,25(OH)₂D₃ that the 1,25(OH)₂D₃ is behaving like the sex hormones and inhibiting IL-2 activity through a genomic effect acting as a classic steroid hormone. At concentrations in excess of 10⁻¹⁰ M 1,25(OH)₂D₃ an effect on the soluble IL-2R is implicated. It is possible that the reason why the message for both IL-2 and IL-2R return to normal could be down regulation of the vitamin D receptor in much the same way as happens in the case of a classic steroid hormone. The increase in soluble IL-2R demonstrated to occur in cells cultured at concentrations of 1,25(OH)₂D₃ greater than 10⁻¹¹ M could mop up the IL-2 produced at these higher concentrations thereby continuing the inhibition throughout the range under scrutiny.

The assessment of the effect of 1,25(OH)₂D₃ on mRNA only accounted for three concentrations (0, 10⁻¹⁰ M and 10⁻⁷ M) of 1,25(OH)₂D₃, it would have been better to look at the range of concentrations (0 to 10⁻⁷ M) to complete the argument. However, this was technically very difficult to achieve because of the large amount of PBMCs required in the extraction process to get adequate mRNA. The findings, however were in agreement with the work of Rigby et al (Rigby, 1987) who demonstrated an inhibition of IL-2 mRNA by 10⁻¹⁰ M 1,25(OH)₂D₃ at 24 hours. However, the effect of 1,25(OH)₂D₃ at any other concentrations was not documented in his work.

CHAPTER 9.

CONCLUSIONS.

It has long been established that $1,25(\text{OH})_2\text{D}_3$ has a role to play in the immune system. The major breakthrough came when it was discovered that $1,25(\text{OH})_2\text{D}_3$ could inhibit IL-2 activity (Tsoukas, 1984) and a role for $1,25(\text{OH})_2\text{D}_3$ as an immunomodulatory hormone was proposed. This was quickly followed by the demonstration that $1,25(\text{OH})_2\text{D}_3$ could inhibit IgG and IgM production (Lemire, 1984). Clearly, the mechanism of $1,25(\text{OH})_2\text{D}_3$ inhibition had to be investigated.

It was proposed that in theory $1,25(\text{OH})_2\text{D}_3$ could inhibit IL-2 activity through the following mechanisms ;

- (1) $1,25(\text{OH})_2\text{D}_3$ could act on the cell to inhibit the production and/or secretion of IL-2 directly and this could possibly be at the level of the mRNA.
- (2) $1,25(\text{OH})_2\text{D}_3$ could act on the IL-2 receptors to increase the consumption of IL-2 thereby lowering the activity of the IL-2.
- (3) $1,25(\text{OH})_2\text{D}_3$ being a differentiation agent could promote the differentiation of suppressor lymphocytes.

These proposals have been addressed in this thesis. Given that $1,25(\text{OH})_2\text{D}_3$ had such a profound effect on the immune system the other calciotropic hormones ($24,25(\text{OH})_2\text{D}_3$, $25(\text{OH})\text{D}_3$, PTH and calcitonin) were studied to ensure that $1,25(\text{OH})_2\text{D}_3$ was unique as far as calciotropic hormones were concerned. The inhibitory effect of $1,25(\text{OH})_2\text{D}_3$ was compared to the documented effect of other steroid hormones in particular the sex hormones (oestrogen, progesterone and dihydrotestosterone) on the immune system (Holdstock, 1982). IL-2 is one of an increasing number of cytokines so the effect of $1,25(\text{OH})_2\text{D}_3$ on IL-1 and IL-6 was also studied. These cytokines could be involved in the particular culture system i.e. the IL-1 and IL-6 are produced by monocytes which were not excluded from the cell preparation.

In chapter 3 the effect of $1,25(\text{OH})_2\text{D}_3$ on lymphocyte transformation was studied and this confirmed that $1,25(\text{OH})_2\text{D}_3$ did inhibit lymphocyte proliferation. The use of different mitogens revealed that $1,25(\text{OH})_2\text{D}_3$ had a greater effect on PHA induced lymphocyte transformation than either Con A or PWM induced lymphocyte proliferation. PHA is predominantly a T-helper cell mitogen and this suggested that the likely target for $1,25(\text{OH})_2\text{D}_3$ may be the T-helper cell. The inhibition was shown to be unique to $1,25(\text{OH})_2\text{D}_3$ in that none of the other calcitrophic hormones ($24,25(\text{OH})_2\text{D}_3$, $25(\text{OH})\text{D}_3$, PTH and calcitonin) exhibited such an inhibition. The effect of sex hormones (oestrogen, progesterone and dihydrotestosterone) was slightly different in that there appeared to be an inhibition of lymphocyte transformation with concentrations up to 10^{-11} M (0 to 10^{-11} M) and at the higher concentrations this inhibition was abolished (10^{-10} M to 10^{-7} M). In this chapter the effect of $1,25(\text{OH})_2\text{D}_3$ was also demonstrated not to be a kinetic effect nor an effect on cell viability. In addition it was demonstrated that $1,25(\text{OH})_2\text{D}_3$ did not effect the activation process. The inhibition of cell proliferation by $1,25(\text{OH})_2\text{D}_3$ was therefore a genuine and unique effect.

In chapter 4 the effect of $1,25(\text{OH})_2\text{D}_3$ and the other calcitrophic and steroid hormones on IL-2 activity was studied. It again transpired that of the calcitrophic hormones ($24,25(\text{OH})_2\text{D}_3$, $25(\text{OH})\text{D}_3$, PTH and calcitonin) only $1,25(\text{OH})_2\text{D}_3$ had a significant inhibitory effect. The effect of the sex hormones (oestrogen, progesterone and dihydrotestosterone) on IL-2 activity was similar to the effect these hormones had on lymphocyte transformation. It was also demonstrated that the inhibition of IL-2 activity was unlikely to arise from a defect in the secretion as lysed cells still exhibited an inhibition by $1,25(\text{OH})_2\text{D}_3$. An inhibition of the activation process by $1,25(\text{OH})_2\text{D}_3$ was also ruled out in this chapter.

Chapter 5 described the effect of $1,25(\text{OH})_2\text{D}_3$ on other cytokines namely IL-1 and IL-6. $1,25(\text{OH})_2\text{D}_3$ had no significant effect on the IL-1 or IL-6 activity. The effect of $1,25(\text{OH})_2\text{D}_3$ on IL-2 activity appeared to be unique to this cytokine.

In chapter 6 the effect of $1,25(\text{OH})_2\text{D}_3$ on the T-cell subsets, T-helper and T-suppressor cells were studied. The $1,25(\text{OH})_2\text{D}_3$ not surprisingly was shown to inhibit both cell proliferation and IL-2 activity in T-helper cells and have no real effect on the T-suppressor cells. This demonstrated that it was very unlikely that the $1,25(\text{OH})_2\text{D}_3$ was promoting the differentiation of T-suppressors as the inhibition still occurred in the absence of T-suppressor cells.

Chapter 7 looked at the effect of $1,25(\text{OH})_2\text{D}_3$ on the sIL-2R concentration. $1,25(\text{OH})_2\text{D}_3$ had the effect of increasing the concentration of the soluble IL-2R in supernatants at concentrations in excess of 10^{-11} M (10^{-10} M to 10^{-7} M). The increase in sIL-2R concentration was an 'all or nothing' response and occurred over the physiological range (10^{-11} M to 10^{-10} M) of the $1,25(\text{OH})_2\text{D}_3$.

In chapter 8 $1,25(\text{OH})_2\text{D}_3$ at 10^{-10} M was shown to inhibit IL-2 production at the mRNA level and also to inhibit the interleukin-2 receptor (IL-2R) production at the mRNA level. It was also demonstrated that if the cells were cultured in the presence of 10^{-7} M $1,25(\text{OH})_2\text{D}_3$ then the message for both IL-2 and IL-2R returned to their original levels.

Taken together the results presented in this thesis would argue for the existence of not one mechanism but two mechanisms for the inhibition of IL-2 by $1,25(\text{OH})_2\text{D}_3$. It can be argued that between the concentrations 0 to 10^{-11} M that the $1,25(\text{OH})_2\text{D}_3$ inhibits IL-2 activity directly i.e. at the level of the message for both IL-2 and its receptor. In other words over this range $1,25(\text{OH})_2\text{D}_3$ is acting as a classic steroid hormone. In the classic steroid hormone situation down regulation of the steroid hormone receptor at the higher concentrations (10^{-10} M to 10^{-7} M) is observed and this leads to the loss of effect (as demonstrated in chapter 3 and 4 by the sex hormones). However, this does not occur when $1,25(\text{OH})_2\text{D}_3$ acts on the immune system. It is at this point that the proposed second mechanism would come into play. At concentrations in excess of 10^{-11} M the inhibition of IL-2 activity is sustained by the increase in the sIL-2R concentration. The sIL-2R acts as an antagonist for the IL-2 effectively mopping up the free IL-2 thus

allowing for the inhibition of IL-2 by $1,25(\text{OH})_2\text{D}_3$ to continue over the range 0 to 10^{-7} M.

Further work has to be carried out to confirm this hypothesis with regard to the postulated down regulation of the vitamin D receptor. However, the effect of the sex hormones on IL-2 activity support this theory in that the IL-2 activity quite clearly recovers and returns to control levels. It is also important to note that the initial inhibition (0 to 10^{-11} M) of the IL-2 activity by the sex hormones (oestrogen, progesterone and dihydrotestosterone) mirrored the inhibition by $1,25(\text{OH})_2\text{D}_3$. The inhibition over the range 0 to 10^{-11} M by $1,25(\text{OH})_2\text{D}_3$, oestrogen, progesterone and dihydrotestosterone of IL-2 activity were indistinguishable from each other.

It is perhaps significant that the switch from one mechanism to the other takes place over the physiological range of the $1,25(\text{OH})_2\text{D}_3$ i.e. between 10^{-11} M and 10^{-10} M. There can now be little doubt that the inhibition of IL-2 activity by $1,25(\text{OH})_2\text{D}_3$ is an important, unique and genuine physiological event.

The significance of having $1,25(\text{OH})_2\text{D}_3$ acting as an immunomodulatory hormone remains unclear. $1,25(\text{OH})_2\text{D}_3$ has long been established as a calcium regulatory hormone functioning primarily in the calcium and phosphorus homeostasis scheme. $1,25(\text{OH})_2\text{D}_3$ is also thought to have a prominent role to play in bone remodelling. It is difficult to see the advantage gained from having the same metabolite acting on the immune system and on the mineral metabolism system. $1,25(\text{OH})_2\text{D}_3$ has also been shown to act as a differentiation factor particularly on the myeloid cell lineage. It is possible that what $1,25(\text{OH})_2\text{D}_3$ does by dampening down the immune response is to facilitate the differentiation of cells down the monocyte/macrophage pathway towards the production of osteoclasts. These osteoclasts could mobilise calcium through bone resorption allowing vitamin D to increase the pool of calcium via both the resorption of bone and the well documented effect of increasing calcium absorption at the intestine.

The potential for using $1,25(\text{OH})_2\text{D}_3$ as an immunosuppressive agent is complicated by the previously mentioned effect on the absorption of calcium from the gut. The administration of quite a small dose of $1,25(\text{OH})_2\text{D}_3$ often introduces a risk of hypercalcemia and this has been borne out in several clinical studies (Cunningham, 1985, and Morimoto, 1986). The solution to these problems lies in finding synthetic analogues to $1,25(\text{OH})_2\text{D}_3$ which will inhibit the immune system but not stimulate gut absorption of calcium and bone resorption.

This approach has already yielded dividends in the management of the common skin disease, psoriasis. In this condition there is abnormal proliferation of squamous epithelial cells with a degree of dedifferentiation of the keratinocytes leading to skin plaques with excessive shedding of keratin scales. Oral administration of $1,25(\text{OH})_2\text{D}_3$ was associated with a degree of hypercalcemia (Morimoto, 1986) but topical application permitted resolution of the plaques without such a marked effect on serum calcium. Even more successful therapeutic results without complications have been obtained using topical application of the analogue calcipotriol.

In some other clinical situations the best approach to achieving satisfactory immunosuppression may be to use $1,25(\text{OH})_2\text{D}_3$ or an analogue in combination with a smaller dose than usual of another more conventional immunosuppressant drug which may have undesirable toxic effects in high dosage. For example the renal toxicity of high doses of cyclosporin A might be ameliorated by employing smaller doses of cyclosporin A in addition to $1,25(\text{OH})_2\text{D}_3$ or analogue.

Preliminary studies of the use of vitamin D metabolites in some types of lymphoma (Cunningham, 1985) ; the demonstration of vitamin D receptors in some breast cancers (Berger, 1987) ; the demonstration of differential survival rates in breast carcinoma relating to the presence or absence of $1,25(\text{OH})_2\text{D}_3$ receptors (Colston, 1989) ; and the effect of $1,25(\text{OH})_2\text{D}_3$ on some aspects of the diabetic patient (Frazer, 1981), all suggest that the effect of $1,25(\text{OH})_2\text{D}_3$ on the immune system may turn out to be an important therapeutic tool. A clearer understanding of the mechanism of this action as described in this thesis is likely to be of considerable relevance.

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